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Editors

Calorie Restriction, Aging and Longevity

Springer

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To my wife, Joyce, with love, and my son Michael,
my daughter Sue, and their families
Wishing everyone healthy, happy and long lives

Arthur

Preface

Food or calorie restriction has been shown in many short-lived animals and the rhesus monkey to prolong life-span. Life-long nutrition studies are not possible in humans because of their long survival. Studies over 2–6 years in healthy adult humans have, however, shown that a 20% reduction in food or calorie intake slows many indices of normal and disease-related aging. Thus, it is widely believed that long-term reduction in calorie or food intake will delay the onset of age-related diseases such as heart disease, diabetes and cancer, and so prolong life.

Over the last 20 or more years there has been a progressive rise in food intake in many countries of the world, accompanied by a rising incidence of overweight and obesity. Thus our increasing food and calorie intake has been linked to the rising incidence of cardiovascular disease and diabetes in early adult life. It is accepted that overeating, accompanied by reduced physical exercise, will lead to more age-related diseases and shortening of life-span. What can be done? Put simply, the answer is to reduce our calorie intake, improve our diet, and exercise more. But calorie restriction is extremely difficult to maintain for long periods. How then can we solve this problem? This book provides the latest information on the beneficial effects of calorie restriction on health and life-span and brings us closer to an understanding at the molecular, cellular and whole organism level of the way forward.

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Part I
Calorie Restriction in Different Species

Chapter 1
History of Caloric Restriction, Aging and Longevity

Edward J. Masoro

Abbreviations

CR	Caloric restriction
DNA	Deoxyribonucleic acid
DR	Dietary restriction
IGF-1	Insulin-like growth factor-1
mRNA	Messenger Ribonucleic Acid
NCTR	National Center for Toxicological Research
NIA	National Institute on Aging

1.1 Introduction

Sustained research on caloric restriction began in 1930 with a study by McCay and his colleagues at Cornell University, which resulted in their seminal publication some 5 years later (McCay et al. 1935). In that study, white rats were used to determine the effect of retarded growth on life span. The protocol of this study involved the following three groups of rats: one group was allowed to grow to maturity at what the investigators considered to be a normal rate; the other two groups were maintained on a reduced energy intake for about 700 and 900 days respectively. As expected the latter two groups grew slowly; however, they lived much longer than those allowed to grow at the normal rate. The authors concluded that retarding the growth of rats extended their life. This finding was in accord with an earlier study by the Cornell group in which the longevity of brook trout was increased when growth was slowed by restricting protein intake (McCay et al. 1929). It should be noted that McCay and associates believed that it was the retarded growth and not the reduction in energy intake per se that was the factor responsible for the life extension. Indeed,

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this hypothesis was the one held by most biological gerontologists for next 40 or more years.

However, it should not be concluded that restricting energy intake had not been explored earlier. In 1914, Francis Peyton Rous, a 1966 Nobel laureate, reported that reducing food intake inhibited the occurrence of spontaneous cancers in rodents (Rous 1914). Since most cancers occur late in life, this finding had gerontological significance. A few years later, Osborne and Mendel, distinguished Yale nutritionists, reported that blunting the growth of female rats by decreasing their food intake led to life extension and the ability to reproduce at older ages than the rats that did not undergo a period of slow growth (Osborne et al. 1917). In the same year, it was reported that reducing food availability extends the life of *Drosophila* (Loeb and Northrop 1917). One might have expected that these findings would have fomented research on the gerontological actions of energy restriction, but such was not the case partly because of the 1920 paper of Robertson and Ray. These workers reported in the prestigious Journal of Biological Chemistry that the rate of growth of mice is positively correlated with the length of life (Robertson and Ray 1920).

Two other important findings were reported in the late 1930s. One was the increased longevity of *Daphnia longispina* induced by the reduction of food intake (Ingle et al. 1937). The other was the further work by the McCay group providing strong evidence that the responsible dietary factor underlying the slow growth was most likely the reduction in caloric intake (McCay et al. 1939). Indeed, in the ensuing years, the term caloric restriction (CR) has been widely used when referring to global food restriction and CR will be the acronym used in this paper.

1.2 The 1940s

Its effects on age-associated diseases dominated CR research in the 1940s. Starting in 1941, John Saxton and his colleagues at Cornell Medical School published papers on the attenuation of age-associated diseases in rats by CR: kidney disease (Saxton and Kimball 1941); and a broad array of solid tumors (Saxton et al. 1948). Clive McCay was a co-author of the 1948 paper; moreover, his group had published an earlier paper on the ability of CR to retard a broad spectrum of spontaneous age-associated diseases in rats (McCay et al. 1943). In 1946, it was reported that the fasting of rats one out of every 4 days extends their lives (Carlson and Hoetzel 1946); A. J. Carlson of the University of Chicago was a leading physiologist of his day. This finding was a forerunner of the every-other-day feeding method of inducing food restriction in use today.

CR was also found to retard age-associated diseases in mice. It inhibited the occurrence of spontaneous tumors (Tannenbaum 1940) and the induction of tumors by chemical carcinogens in mice (Tannenbaum 1944). It was also reported that CR inhibits the development of leukemia in mice (Saxton et al. 1944). The laboratory of Maurice Visscher, the distinguished University of Minnesota physiologist, reported that spontaneous mammary tumors are retarded in mice by CR (Visscher et al. 1942). The ability of methylcholanthrene to induce mammary tumors in mice was also found to be inhibited by CR (White et al. 1944). In addition to the cancer work, Visscher's group showed that CR maintains fertility of female mice to very advanced ages (Ball et al. 1947); thus, the early findings on rats of Osborne and Mendel were expanded to include mice.

1.3 The 1950s

Publications on CR were sparse during this decade. The Tannenbaum group continued to publish its work on the effect of CR on carcinogenesis in mice (Tannenbaum and Silverstone 1953) as did the Visscher group (Lee et al. 1952). The Visscher group also further explored the effects of CR in retarding reproductive senescence in mice (Visscher et al. 1952).

The report (Rudzinka 1951) that food restriction enhances the longevity of the protozoan (*Tokophrya infusionum*) and the study (Chvapil and Hruza 1959) showing that CR retards the age-associated increase in rat tail collagen cross-linking were the only truly significant new advances in the 1950s. Also, in the late1950s, Morris Ross of the Fox Chase Cancer Institute reported on the beginning of his group's monumental studies on the effects of CR in rats (Ross 1959).

1.4 The 1960s

In 1960, it was hypothesized that CR extends life by reducing body fat content (Berg and Simms 1960); this was the first challenge to the retardation of growth hypothesis proposed some 25 years earlier by McCay and colleagues. Although Berg and Simms had not measured body fat content, they assumed that CR would decrease it, a reasonable assumption. The basis of their hypothesis was the growing evidence that obesity is a health risk in humans. Most of the gerontological community was not impressed with this hypothesis and continued to subscribe to McCay's view. Although nutritionists did embrace the hypothesis of Berg and Simms, they found the life-extending action of CR to be of little conceptual interest since they viewed it as merely a procedure for decreasing body fat content.

It was during the 1960s that the Ross group made their major contributions to CR field. Much of their work on CR such as the retardation of kidney disease (Bras and Ross 1964) and of tumors (Ross and Bras 1965) confirmed findings published in earlier years by other investigators. However, the quality of their studies made believers out of doubters. Indeed, in 1973, I heard Morris Ross present the findings of his group at a National Institutes of Health workshop held in Seattle and was so impressed that I spent most of the remainder of my research career studying CR. This group was also the first to determine the effect of CR on the age-specific death rate of rats and found that CR decreased it from ages 6 through 24 months (Ross 1961).

In the 1960s, it was reported that CR extends the life of rotifers (Fanestil and Barrows 1965) and a fish species (*Lebistes reticulates*) in captivity (Comfort 1963). Also, measurements of enzymatic activities started to be used for the exploration of the biochemical basis of the actions of CR (Barrows and Roeder 1969). This was first use of a then cutting edge tool of biochemistry in the study of CR. Although enzymatic analyses have often been employed in the ensuing decades, surprisingly these analyses have provided little understanding of the mechanisms underlying the life-extending and related actions of CR.

1.5 The 1970s

Based on his extensive studies on the effects in rats of hypophysectomy and/or CR, Arthur Everitt proposed in the early 1970s that pituitary function and CR are linked. In 1970, he considered the link to be the functioning of the pituitary in the regulation of food intake (Everitt 1970). However, by 1973, he viewed the link to be a CR-induced decrease in the secretion of an aging factor by the pituitary (Everitt 1973). This latter view was a forerunner of the current debate on a possible role of the Growth Hormone-IGF-1 axis in the life-extending action of CR. Over the next three decades, Arthur Everitt and his colleagues contributed to our knowledge of CR in the areas of morphology, physiology, and pathology.

In the 1970s, Roy Walford and colleagues began to publish their extensive work on the actions of CR reporting its effects on immune function in mice (Walford et al. 1973). The studies of this group continued for the next three decades and covered a range of physiological and biochemical subject areas. Richard Weindruch was a product of the Walford laboratory and he has gone on to a distinguished independent career in CR research, some of his important findings and concepts will be presented in the discussion of the 1980s and 1990s.

In the 1970s, Robert A. Good and colleagues published their initial work on the beneficial effects of CR in mice with autoimmune diseases (Fernandes et al. 1976). These studies continued for the next two decades in Good's laboratory and then independently in the laboratory of Gabriel Fernandes well into the twenty first century.

In 1977, it was reported that food restriction extends the life of the nematode, *Caenorhabditis elegans*, (Klass 1977). This species has since become a major animal model in CR research.

It was in 1977 that George Sacher proposed that CR retards aging by reducing the metabolic rate per unit of body mass (Sacher 1977). Although Sacher had not measured the metabolic rate, this hypothesis rapidly gained favor replacing McCay's retardation of growth hypothesis as the dominant view held by biological gerontologists.

In 1979, Brian Merry and Ann Holehan published the first of their extensive work on the effect of CR in retarding reproductive senescence in the rat model (Merry and Holehan 1979). Merry went on in the 1980s and 1990s to contribute broadly to the knowledge base of CR in several areas of biochemistry.

1.6 The 1980s

The validity of the three major hypotheses on the mechanism underlying the life-extending action of CR was challenged by studies reported early in the 1980s. Walford's group found that CR initiated at 12 months of age significantly extends the life of mice (Weindruch and Walford 1982) and our group reported that male F344 rats, which have essentially full skeletal growth by 6 months of age, exhibit almost the same increase in life span when CR is initiated at 6 months of age as when started at 6 weeks of age (Yu et al. 1985). These two studies spelled the end of the long period of belief in McCay's retardation of growth hypothesis.

Data dissociating the life extending action of CR from its ability to decrease body fat content was reported by our group for rats (Bertrand et al. 1980) and David Harrison's group for mice (Harrison et al. 1984). For a while this spelled the end of belief in the reduction of body fat hypothesis of Berg and Simms. However, in the twenty first century that hypothesis has been resurrected by investigators, who have ignored the findings of the 1980s rather than providing contrary evidence (Bluher et al. 2003).

Our group reported that over most of their life span, food intake per gram body mass is greater in rats on a CR regimen than in rats fed ad libitum*;* moreover, when the data are expressed per gram lean body mass, there is no difference between the two groups (Masoro et al. 1982). These findings are not in accord with Sacher's reduction of metabolic rate hypothesis. The then many advocates of Sacher's view believed so strongly in his hypothesis that this contrary evidence was ignored at the time by most of them.

During the 1980s, our group used semi-synthetic diets to test the validity of the claim that the dietary factor underlying the life-extending action of global food restriction is the reduced intake of energy. We found that the reduced intake of protein (Masoro et al. 1989), fat (Iwasaki et al. 1988), minerals (Iwasaki et al. 1988), and vitamins (Yu et al. 1982) did not underlie the life extending action. These studies provided further strong evidence for the claim of McCay and colleagues that the reduced intake of calories is the responsible dietary factor. Surprisingly, this evidence is frequently ignored by the authors of papers published in the twenty first century, who refer to the life extending and anti-aging actions of global food restriction by the ambiguous term, dietary restriction (DR) rather than CR.

During the 1980s, George Roth, Donald Ingram, James Joseph, and other colleagues in the intramural program of the National Institute on Aging (NIA) studied the effects of CR on the nervous system function including behavior. One of the most significant of their many studies is the finding that CR delays in rats the age-associated loss of striatal dopamine receptors (Roth et al. 1984).

In 1985, Brian Merry's group reported that CR increases whole body protein turnover in rats 1 year of age and older (Lewis et al. 1985). This finding suggested that CR may retard aging by decreasing the age-associated cellular accumulation of damaged proteins.

During the 1980s, Dike Kalu and his associates intensively studied the effects of CR on bone. They found that CR prevents senile osteopenia in male F344 rats (Kalu et al. 1984). And Arthur Everitt and coworkers reported that CR markedly decreases the prevalence of hind leg paralysis in rats at advanced ages (Everitt et al. 1985).

It was in the 1980s that Arlan Richardson and his colleagues pioneered the use of the technology of molecular biology in CR research reporting studies on the effects of CR on hepatic mRNA (Birchenall-Sparks et al. 1985), the expression of the $\alpha_{2\upsilon}$-globulin gene (Richardson et al. 1987), and the repair of DNA (Weraarhakul et al. 1989). Since then, Richardson has continued to utilize the developing tools of molecular biology in the study of CR. This technology now dominates research on CR.

In the 1980s, two USA government agencies, the National Institute on Aging (NIA) and the National Center for Toxicological Research (NCTR), jointly established a program for the generation and study of a number of rat and mouse strains maintained on a CR-diet along with animals of the same species and strain that were fed ad libitum. This program, directed by Ronald Hart and Angelo Turturro had two functions: intramural studies and the distribution of animals for research on CR by investigators at academic and other research institutions. The intramural studies were focused on longevity and pathology along with studies in the areas of expertise of the NCTR staff: physiology (Phillip Duffy), intermediary metabolism (Ritchie Feuers), and drug metabolism (Julian Leakey). The intramural program provided a wealth of important information, particularly in regard to comparing the effects of CR on longevity and pathology in a spectrum of rat and mouse genotypes. Its extramural function of providing animals to investigators with expertise in many different biological areas proved to be even more valuable; by this means, it greatly expanded the participation of leading biologists in CR studies as well as aging research in general.

Late in the 1980s, Richard Weindruch and Roy Walford co-authored an encyclopedic book on what was known about CR at that time (Weindruch and Walford 1988). This book had a major impact in interesting investigators in a variety of biological disciplines in the study of aging and, in particular, CR.

In 1989, two concepts were proposed regarding the evolutionary basis of the life-extending action of CR. One was based on the selection of organisms that respond to a scarcity of food by decreasing the rate of reproductive aging (Harrison and Archer 1989) and the other was based on the selection of organisms that divert energy usage from reproduction to somatic maintenance when food availability is limited (Holliday 1989). The latter view has gained wide acceptance. Also in that year, Steven Austad reported that CR extends the life of the bowl and doily spider, *Frontinella pyramitela* (Austad 1989) and Allen Taylor and colleagues reported that CR delays cataract formation in the Emory mouse (Taylor et al. 1989).

1.7 The 1990s

In 1990, initial findings were reported from the NIA intramural research program started in the late 1980s on the effects of CR on non-human primates (Ingram et al.

1990). In 1993, the initial findings of the University of Wisconsin study on the effects of CR on rhesus monkeys were published (Kemnitz et al. 1993) and in that same year, investigators at the University of Maryland interpreted findings of their ongoing study of rhesus monkeys in terms of CR (Hansen and Bodkin 1993). These studies have continued through the 1990s and into the twenty first century; many of the findings on physiological processes in these primates are similar to those observed with rodent models. Importantly, CR in the non-human primate models has been found to decrease risk factors that have links to age-associated diseases in humans. As of yet, the longevity data from these studies have not been of a quality to confidently claim that CR extends the life span of the primate species studied. In the 1990s, findings were reported on the effect of CR on another primate species, *Homo sapiens* (Walford et al. 1992). Roy Walford was a member of the Biosphere 2 crew. This crew attempted to grow their own food, which resulted in an unplanned for CR during most of their time in Biosphere 2; Walford made physiological and biochemical assessments on the crew and found the effects of a low food intake to be similar to what occurs in rodent species. These findings were a forerunner to the excellent research on CR in humans being done in the twenty first century (Fontana et al. 2004).

It was known that the plasma levels of corticosterone, the major glucocorticoid in rodents, are transiently increased upon initiating CR. In 1991, it was shown that the daily peak concentration of plasma free corticosterone remains elevated throughout long-term CR (Sabatino et al. 1991). This is an important finding because free corticosterone is the biologically active form of glucocorticoid in the rat and glucocorticoids play a key role in enabling mammals to cope with harmful environments.

In 1992, it was reported that the metabolic rate per unit of fat-free mass or per unit of metabolic mass is similar in ad libitum-fed rats and those on a CR regimen over most of the life span (McCarter and Palmer 1992). One might think that this finding would have put an end to the belief in the hypothesis that CR retards aging by decreasing the metabolic rate. However, that was not the case. Rather this finding fostered what is still an ongoing debate on how to normalize findings on metabolic rate for differences in body size.

It was also found that daily rate of glucose fuel use per unit of metabolic mass is the same for rats on a CR regimen as for rats fed ad libitum; this was somewhat surprising finding since the mean 24 h plasma level of glucose is modestly decreased and plasma insulin level is markedly lower in rats on a CR regimen (Masoro et al. 1992). These findings strongly indicated that CR enhances insulin sensitivity. Indeed, it recently has been claimed that the increase in insulin sensitivity plays an important role in the life-extending action of CR (Bonkowski et al. 2006). However, it should be noted that the evidence in support of this claim is less than robust.

In the 1990s, William Sonntag and his colleagues broadly examined the effects of CR on the growth hormone-IGF-1 axis; they measured growth hormone secretion, plasma IGF1 levels, and IGF1 receptors and other factors. At the end of the decade, this group published a paper summarizing the gerontological significance of their extensive work (Sonntag et al. 1999). It would be wise for current investigators studying the role of IGF-1 in aging and CR to familiarize themselves with the studies of the Sonntag group.

Several new hypotheses were posed during the 1990s on the mechanisms underlying the life extending and related actions of CR. It was proposed that CR retarded aging by enhancing apoptosis thereby retarding the accumulation of damaged cells (Warner et al. 1995). Indeed, there is evidence that dysregulation of apoptosis (inadequate as well as excessive) occurs during aging and that CR mitigates these problems.

In 1996, Sohal and Weindruch (1996) and independently Yu (1996) asserted that CR extends life by attenuating oxidative damage. Currently, this hypothesis is the one favored by most biological gerontologists. Indeed, it is clear that CR retards the age-associated accumulation of cellular oxidative damage. However, it has yet to be established that oxidative damage plays a major role in the aging process.

Also in 1996, CR-induced reduction of body temperature was postulated to be a mechanism underlying life extension and retardation of aging (Koizumi et al. 1996). This view was supported by mouse studies carried out in the authors' laboratory and by the fact that in several poikilothermic species reducing the environmental temperature extends life (Finch 1990). However, the validity of this view is countered by the fact that the magnitude of CR-induced life extension is similar in mice and rats even though CR reduces body temperature much more markedly in mice than in rats.

In 1998, I proposed the hormesis hypothesis (Masoro 1998) and in that same year, investigators working on CR at the NCTR independently did, too (Turturro et al. 1998). This hypothesis provides a broad framework for studies aimed at understanding the complex nature of the processes that underlie the anti-aging actions of CR. At first, few others embraced this view. However, during the twenty first century it has gained supporters, probably because it appears increasingly likely that an interaction of multiple processes underlies the life-extending and anti-aging actions of CR.

1.8 The Twenty First Century

It does not seem appropriate to discuss twenty first century findings in historical terms. Of course much of the research in the first decade of the twenty first century has been a continuation of investigations begun in the twentieth century. For example work started in the twentieth century on nonhuman primates is being completed and extended and excellent new studies on the human primate have been undertaken. The major mechanistic innovation so far in the twenty first century is the extensive exploration of potential signaling pathways involved in the actions of CR and the interactions among these pathways. These studies will require the development of innovative ways of testing hypotheses generated from such complex data sets. However, predictions about where science is going are notorious for their ineptitude.

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Chapter 2
Food Intake, Life Style, Aging and Human Longevity

Arthur V. Everitt, Leonie K. Heilbronn, and David G. Le Couteur

2.1 Calorie Restriction and Human Survival

Since the 1980s, the prevalence of obesity in the USA has risen steadily (Fig. 2.1). In the fifteenth century an Italian nobleman Luigi Cornaro lived a life of gluttony until middle age. When his health started to fail his physician advised him to eat less, his health improved and he lived to 102 years. Cornaro (Fig. 2.2) argued that dietary restriction and moderate living lead to good health and long life (Cornaro and Butler 1903; Cornaro and Howell 1987; Howell and Cornaro 1987; Olshansky 2006). In was not until the mid-twentieth century that Breslow (1952) in United States reported that overweight people had higher death rates than those of normal weight.

The classic calorie restriction (CR) studies first performed by McCay (1935), showed that long term 40% CR without malnutrition reduces body weight and prolongs the life of the laboratory rat. Whether the longevity-enhancing effects of food restriction are secondary to reduced intake of calories or some specific nutrient such as fat or protein is still under discussion and may vary with the species (Masoro 1998; Anderson and Moore 2004; Mair et al. 2005; Piper et al. 2005; Simpson and Raubenheimer 2007; Lee et al. 2008). In CR, the intake of calories is reduced, but sufficient vitamins, minerals and other essential nutrients must be eaten. CR retards many physiological aging processes and delays the onset and progression of most diseases of old age in rodents (Berg and Simms 1960; Ross and Bras 1971; Weindruch and Walford 1988; Masoro 2005). Since the maximum life duration of the rat is about 3 years, longevity studies may be completed in 5 years. In a primate, the rhesus monkey, 20 years of CR was found to delay disease onset and prolong life (Colman et al. 2009). However in humans, because the lifespan is more than 100 years it has not been possible to carry out well-controlled life-long studies.

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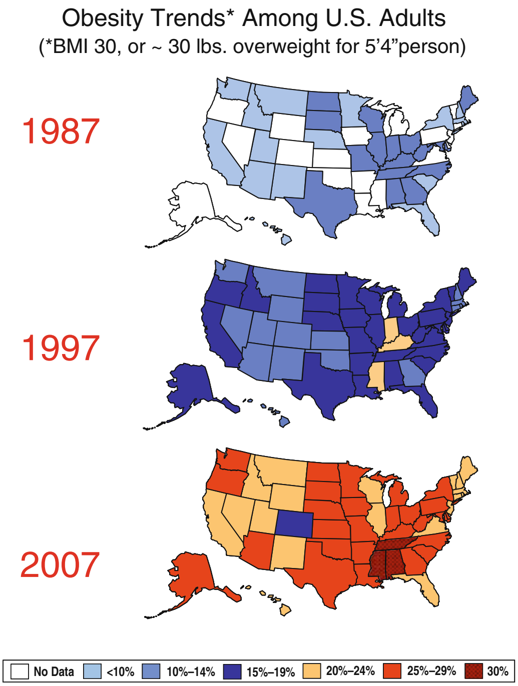
Obesity Trends* Among U.S. Adults
(*BMI 30, or ~ 30 lbs. overweight for 5'4"person)
1987
1997
2007
☐ No Data
<10%
10%–14%
15%–19%
20%–24%
25%–29%
30%

Fig. 2.1 Mapping showing the increased prevalence of obesity across each of the states of the USA between 1987 and 2007 (from Centre for Disease Control and Prevention http://www.cdc.gov/nccdphp/dnpa/obesity/trend/maps/index.htm accessed March 2009)

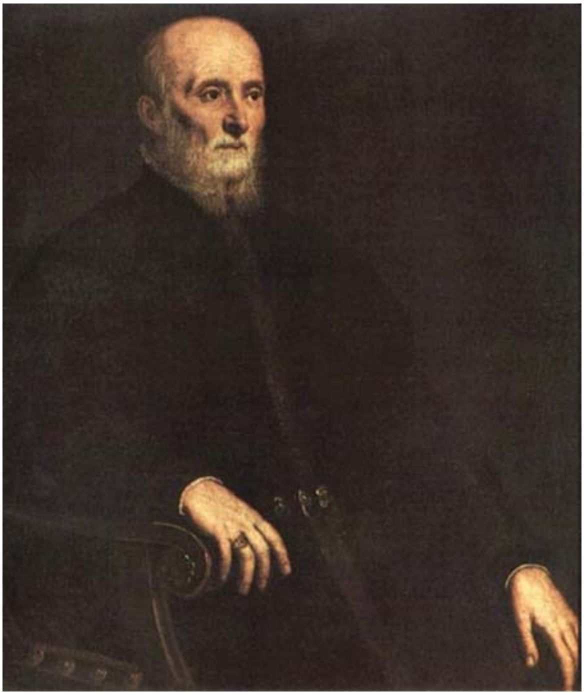

Fig. 2.2 Luigi Cornaro was a sixteenth century nobleman living in Genoa. At age 35 he became sick from overeating. His doctor told him to cut out the rich food and eat as little as you can. Within a few days Luigi recovered and eventually lived to 102. Luigi wrote books extolling the benefits of eating less food. His most famous work is "The Art of Living Long" published by Springer in 2005, in the book of Butler, R. and Olshansky, S.J. (This picture of Cornaro is from a portrait by Jacopo Robusti Tintoretto, c1560–62)

There are however, a number of times in recent history where food availability has been restricted. For example, the Second World War reduced the food intake of many living in Europe and this appears to have had some anti-aging benefits. In the 1940s Scandinavians were surviving on a semi-starvation diet equivalent to about 20% CR and subsequently were found to have a reduced incidence of cardiovascular disease (Strom and Jensen 1951). In Northern Europe during the Second World War, there were fewer cases of hypertension, heart disease and diabetes (Van Itallie and Hirsch 1979).

A comparison of health and mortality in Okinawa and mainland Japan revealed differences in pathology, survival and the incidence of centenarians probably related to calorie intake (Kagawa 1978). The calorie intake of children on Okinawa was only 62% of the intake for Japan, and for adults 80% (Hokama et al. 1967). The mortalities in Okinawa from cerebrovascular disease, cancer and heart disease were 59, 69 and 59% of the rates in the rest of Japan. Thus, the Okinawans appear to have undergone a mild CR over the last 50 years that has led to a small increase in their life expectancy (Willcox et al. 2006b) and Okinawa has the highest rate of centenarians in the world (Willcox et al. 2008b). The mortality rate of Okinawans aged 60–64 is 50% lower than that of Americans and it has been proposed that this is because Okinawans are eating a low calorie diet composed mainly of vegetables, grains, fruit, soy and fish (Dirks and Leewenburgh 2006; Willcox et al. 2007). Okinawans, the longest-lived people on earth eat 40% fewer calories than the Americans, have a life-long low BMI, and live 4 years longer (Willcox et al. 2006b; Willcox et al. 2007). Since Okinawans differ from Americans in genetics, diet and lifestyle, many factors other than calories may contribute to differences in survival.

2.2 Human Trials of Calorie Restriction

During the last 20 years there has been an explosion of interest in research on aging in human subjects. There have been a large number of reviews discussing the probable effects of CR on human aging, age-related disease and longevity (Heilbronn and Ravussin 2003; Masoro 2005; Dirks and Leeuwenburgh 2006; Hunt et al. 2006; Willcox D et al. 2006b; Yu 2006; Everitt and Le Couteur 2007; Holloszy and Fontana 2007; Roberts and Schoeller 2007; Fontana 2008; Mair and Dillin 2008; Redman et al. 2008; Ungvari et al. 2008; Fontana 2009a).

The main aim of aging research in humans has been to reduce morbidity and delay mortality in the elderly (Schneider et al. 1986). In United States in the late 1940s Ancel Keys studied the effects over 6 months of a 40% reduction in food intake of 32 lean men causing malnutrition and producing large reductions in body functions (Keys et al. 1950). This pioneering study showed that CR lowered body weight, pulse rate, body temperature and both systolic and diastolic blood pressures, which increased when CR stopped. However, the diet in this study was of poor quality and had adverse psychological effects. From his Seven Countries epidemiological studies Ancel Keys linked low food intake to decreased blood cholesterol levels and reduced incidence of heart attacks (Keys 1980).

In Spain in the 1950s a 3 year study of nursing-home residents compared 60 randomly assigned non-obese men on 35% CR with 60 men on a normal diet (Vallejo 1957; Stunkard 1976). In this study CR subjects received one liter of milk and 500 g fruit on alternate days. CR subjects spent 123 days in the infirmary compared with 219 days in controls, but there was no significant difference in death rate (6 vs. 13).

In the 1990s and early 2000s 20% CR was shown over periods of 2–6 years in healthy adults to reduce the risk factors for cardiovascular disease and diabetes, major causes of death (Walford et al. 2002; Fontana et al. 2004). Although these human studies of CR ran only for up to 6 years they showed health benefits very similar to those seen in CR rats and mice over their lifetime (Holloszy and Fontana 2007). The first study that started on 26 September 1991, was led by Roy Walford (Fig. 2.3) on eight healthy adult humans (four men and four women) over 2 years in the Biosphere 2, a closed ecological space in Arizona, United States.

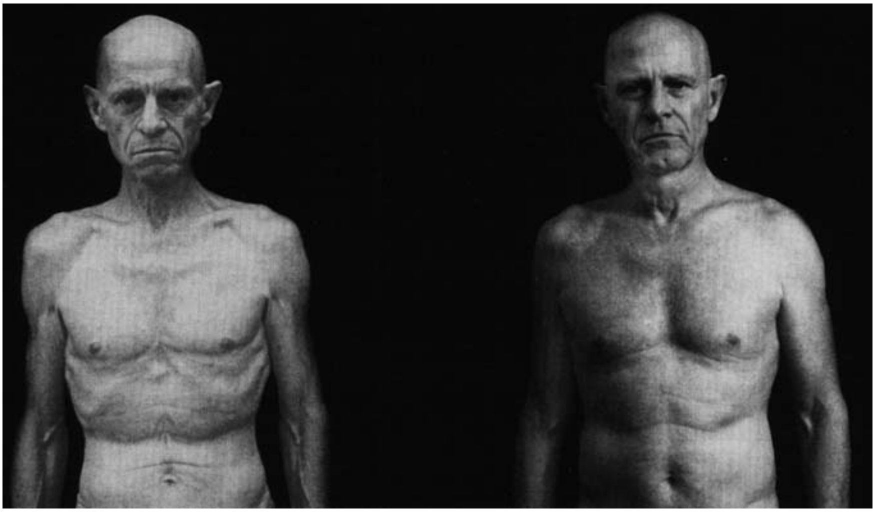

Fig. 2.3 Roy Walford after 15 months of 20% CR (*left*) with body weight 54 kg and after exiting Biosphere 2 with body weight 68 kg. (Adapted from Walford et al. 2002 with permission of the Gerontological Society of America)

This study inadvertently led to a 20% CR over the 2 years and clearly revealed anti-aging effects on many physiological parameters (Walford et al. 1992; Walford et al. 2002). These studies recorded significant reductions of 20% or more in body weight, blood pressure (Fig. 2.4), blood cholesterol and blood sugar, major risk factors for cardiovascular disease and diabetes.

A retrospective cross-sectional analysis was carried out on 18 members of the Calorie Restriction Society (three women and 15 men) average age 50 years with self-reported CR over a period of 6 years on average, with 18 age-matched controls eating a typical American diet. All were non-smokers in good health and none had

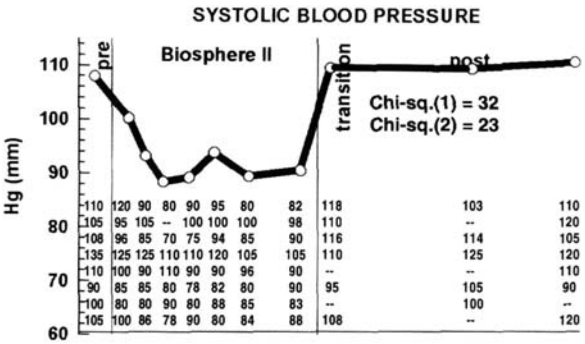

Fig. 2.4 Systolic blood pressures of 8 subjects in the Biosphere 2 study of 20% CR, recording individual values, graphing averages over 24 months and showing the return to normal (Adapted from Walford et al. 2002)

chronic disease (Fontana et al. 2004; Holloszy and Fontana 2007). The CR group members were leaner with a body mass index (BMI) of 19.6 vs. 25.9 in controls and had lower values (Table 2.1) for total cholesterol, LDL cholesterol, triglycerides, systolic and diastolic blood pressures, fasting plasma insulin and glucose (Holloszy and Fontana 2007). Caution must be exercised when interpreting this study however, as this is a retrospective analysis of self-reported CR and the control group selected whilst typical of the average American, was on average overweight.

Table 2.1 Effects on atherosclerosis risk factors of 20% CR over 6 years in 18 healthy 50 year old humans compared with 18 same aged controls (Fontana et al. 2004)

Parameter	CR (*n*=18)	Controls (*n*=18)	*P* value
Tchol mg/dl	158±39	205±40	0.001
LDL-C mg/dl	86±28	127±35	0.0001
HDL-C mg/dl	63±19	48±11	0.006
Tchol/HDL-C ratio	2.6±0.5	4.5±1.3	0.0001
TG mg/dl	48±15	147±89	0.0001
TG/HDL-C ratio	0.8±0.3	3.5±2.8	0.0001
Systolic BP mmHg	99±10	129±13	0.0001
Diastolic BP mmHg	61±6	79±7	0.0001
Fasting glucose mg/dl	81±7	95±8	0.0001
Fasting insulin mIU/ml	1.4±0.8	5.1±2	0.0001
Hs-CRP µg/ml	0.3±0.2	1.6±2.2	0.001

The CR group also had significantly lower levels of inflammation as shown by reduced values for C-reactive protein (Fontana et al. 2004). Inflammation is commonly associated with many chronic diseases including cardiovascular disease (Yu 2006). Long-lived people have a decreased risk of inflammation (Mazziotti et al. 2006). The studies of Fontana (2008, 2009b) indicate that a high energy intake and adiposity cause systemic inflammation, whereas CR has a potent anti-inflammatory effect.

Other studies have shown that low values of cardiovascular and diabetes risk factors as well as other physiological predictors in middle age at about 50 years are associated with an increased probability of a long and healthy life (Manton and Vaupel 1995; Terry et al. 2005; Lloyd-Jones et al. 2006; Willcox B et al. 2006a). A 25-year follow-up from the Baltimore Longitudinal Study of Aging showed that healthy men who displayed three biomarkers of the CR phenotype (lower insulin levels, lower body temperature and slower decline in levels of dehydroepiandrosterone sulphate) had significantly longer survival (Roth et al. 2002). This study also showed that basal metabolic rate (BMR) declined with age and the rate of decline accelerated at older ages (Rizzo et al. 2005). Further, it was also shown that a high BMR is a risk factor for mortality (Ruggiero et al. 2008).

CR appears to retard primary aging of the heart, greatly slowing the decline in diastolic function and reducing markers of inflammation (Meyer et al. 2006). Fontana's research showed that 6 years of CR reduced the development of atherosclerosis, the major cause of mortality in old age. The CR group had 40% lower values of carotid artery intima-media thickness (Fontana et al. 2004). In the CALERIE studies at Pennington, Heilbronn et al. (2006) clearly showed that 6 months of 25% CR reduced deep body temperature and plasma insulin, which were earlier found to be biomarkers of longevity (Roth et al. 2002). In the Washington University CALERIE study, 12 months of 10% CR resulted in a 10% decrease in body weight, reductions in BMI and body fat levels (Racette et al. 2006). In a 36 year follow-up study of healthy non-smoking Japanese–American men (Willcox et al. 2004) the lowest risk for all-cause mortality occurred in those who consumed a modestly low-energy intake (85% of the group mean).

2.3 Human Studies with CR Alternatives

Although CR is very difficult to maintain for long periods there is a Calorie Restriction Society in United States whose members are practising long-term CR, which appears to be slowing some aspects of their aging process (Table 2.1; Fontana et al. 2004). Nevertheless it is unlikely that humans in general will undertake lifelong CR. It is more likely that CR of a lesser degree such as every-other-day feeding would be more acceptable (Dirks and Leeuwenburgh 2006; Varady and Hellerstein 2007). In this routine a feed day is alternated with a fast day. Whilst only short term studies (without CR control groups) have been conducted in normal weight humans to date, alternate-day CR promises to be a good method of weight control, and improves biochemical findings such as improving insulin sensitivity and increasing tyrosine phosphorylation of IRS-1, and slows age changes in a similar manner to CR (Heilbronn et al. 2005; Johnson et al. 2007; Varady and Hellerstein 2007). There is as yet no evidence whether this type of diet will work in overweight or obese individuals. In rodents, there is also evidence that alternate day fasting shifts fat distribution to a healthier phenotype. Whilst body weights were lower after 4 weeks in mice randomized to CR versus alternate day fasting, a similar decrease in the proportion of visceral fat and increase in adiponectin was observed (Varaday et al. 2009). This is important as upper body obesity or central fat is associated with much higher risk of insulin resistance and diabetes, cardiovascular disease and cancer (Cornier et al. 2008; Despres et al. 2008; Fontana 2008).

Another approach would be to use CR mimetics to achieve the same or even greater pro-longevity effects (Ingram et al. 2004; Ingram et al. 2006). These may target metabolic and stress pathways affected by CR such as hormones, nutraceuticals, pharmaceuticals, or genetic manipulations (Dirks and Leeuwenburgh 2006). Possible CR mimetics include drugs that inhibit glycolysis (2-deoxyglucose), enhance the action of insulin (metformin) or affect stress pathways such as resveratrol (Sinclair 2005; Ingram et al. 2006).

Other methods of slowing aging and reducing mortality are to adopt a healthy lifestyle of maintaining a lean body weight, being a non-smoker, eating a healthy diet (fruit, vegetable, whole grains, fish), and being physically active (Goldberg et al. 1996; Knoops et al. 2004; Spencer et al. 2005; van Dam et al. 2008). Physical activity has similar actions as CR on aging changes (Poehlman et al. 2001; Fontana et al. 2007). Thus, a combination of physical exercise and CR may make it easier to achieve the anti-aging effect.

2.4 Physical Exercise and Longevity

Greater physical activity to utilize excess calories has been shown in rats to increase mean lifespan, but not the maximum lifespan (Holloszy et al. 1985). The early study of Pekkanen and colleagues (1987) of 636 middle-aged Finnish men over 20 years showed that high physical activity was associated with decreased mortality. In a later study in Texas of 25,341 men of average age 42.9 years followed over 19 years, increased physical activity was found to improve the quality of life and significantly reduce the all-cause mortality (Kampert et al. 1996). Physically active men, but not women, were at a lower risk of mortality than sedentary ones (Kampert et al. 1996). Data from the Framingham Heart Study show that men and women with moderate physical activity and no diabetes at age 50 years lived 2.3 years longer than those who were sedentary. High physical activity increased the life extension to 4.2 years (Jonker et al. 2006). Again the Framingham Heart Study data show that moderate levels of physical activity during adulthood can add 1.5–3.5 years to life expectancy in men and women (Franco et al. 2005). In a 1-year controlled trial on middle-aged men and women of normal weight, CR and exercise-induced negative energy balance produced similar improvements in risk factors for coronary heart disease (Fontana et al. 2007). Mortality in old age is reduced by energy expenditure in physical activity (Hakim et al. 1998; Manini et al. 2006; Carlson et al. 2007; Kruk 2007; Landi et al. 2008). Landi et al. (2008) found that walking for only 1 h per day in old age was able to reduce mortality. However, one study showed that a high level of physical activity did not compensate for the excess mortality due to overweight and obesity (Orsini et al. 2008).

2.5 Nutrition and Longevity

While there are no lifelong CR studies in human populations, nutritional status is a predictor of mortality even in old age (Haveman-Nies et al. 2002; Michels and Wolk 2002; Haveman-Niess et al. 2003; Hu 2003; Kant et al. 2004; Knoops et al. 2004; Gonzales et al. 2008; Heidemann et al. 2008). It has been proposed that a healthy diet should consist of fatty fish rich in omega-3 fatty acids which are anti-inflammatory, 5 servings a day of fruit and vegetables that are anti-oxidant, whole grains to lower blood cholesterol, yoghurt and nuts containing B vitamins for heart and brain (Michels and Wolk 2002; Haveman-Nies et al. 2003; Hu 2003; Tucker et al. 2005). A prospective study over 18 years in 72,113 healthy women compared effects on mortality (Heidemann et al. 2008) of a high prudent diet (high intakes of vegetables, fruit, legumes, fish, poultry and whole grains) with a Western diet (high red meat, processed meat, refined grains, French fries, and sweets/desserts). The high prudent diet was associated with a 28% lower risk of cardiovascular mortality and a 17% lower risk of all-cause mortality (Heidemann et al. 2008).

There are follow-up investigations assessing the effects over 10–15 years of Mediterranean diets on all-cause mortality in elderly subjects aged 70–90 years (Lasheras et al. 2000; Knoops et al. 2004; Mitrou et al. 2007; Brunner et al. 2008). Those who adhere to the Mediterranean diet had a lower mortality (Tricopoulou et al. 2003; Tricopoulou 2004; Tricopoulou and Critselis 2004). The Mediterranean diet is low in calories and includes plenty of fruit and vegetables (but not potatoes), olive oil or canola, small portions of nuts, fish eaten regularly, red wine in moderation, and very little red meat (Trichopoulou 2001; Mitrou et al. 2007). Olive oil characterizes the diet and allows the consumption of vegetables and salads. Virgin olive oil provides large amounts of stable and not easily oxidisable fatty acids (Battino and Ferreiro 2004). Subjects in these studies were non-smokers and physically active. Thus Mediterranean diets decreased the risks of diabetes, heart attacks and strokes; and reduced the all-cause mortality in the elderly up to age 90 (Trichopoulou 2004). In view of the risks of calorie restriction in the elderly (Dirks and Leeuwenburgh 2006; Fontana and Klein 2007) the Mediterranean diet may be an alternative healthy lifestyle leading to life extension in the elderly.

Vegetarian diets consumed by a cohort of 34,192 white Californian Seventh Day Adventists followed up from 1976 to 1988 are reported to increase life expectancies at age 30 years by 4.42 years in women and 7.28 years in men (Fraser and Shavlik 2001). The health benefits of fruit and vegetable diets have been recognized for some time (Bazzano et al. 2002; Bazzano et al. 2003; Genkinger et al. 2004; Hung et al. 2004; Schneider et al. 2007; Gonzalez et al. 2008). The energy intake of vegetarians is lower than that of non-vegetarians (Woo et al. 1998; Fraser and Shavlik 2001).

In a prospective study of 288 elderly people aged 60–85 years in Spain a high fruit intake reduced mortality, while potato consumption shortened life (Gonzales et al. 2008). There is evidence that having a vegetable-rich diet in childhood is associated with a healthy diet in old age (Maynard et al. 2006). Foods of plant origin seem to be more beneficial in chronic disease prevention than meats (Sabate 2003). Low meat consumption may increase life expectancy in humans (Singh et al. 2003).

Although a diet rich in antioxidants appears to correlate with health, the use of antioxidant supplements is now being discouraged because of increased mortality risk (Bjelakovic et al. 2008). Antioxidant vitamin supplementation has been shown in a number of long-term studies to increase mortality in smokers and participants of the SENECA study (Brzozowska et al. 2008), in chemotherapy and radiotherapy (Lawenda et al. 2008), in cardiovascular disease (Sesso et al. 2008) and in cancer (Bjelakovic et al. 2008; Lin et al. 2009).

2.6 Healthy Lifestyle and Longevity

The so-called healthy lifestyle factors are also associated with reduced mortality (Goldberg et al. 1996; Haveman-Nies et al. 2003; Knoops et al. 2004; Spencer et al. 2005; King et al. 2007; van Dam et al. 2008). Intelligence enhances the individual control over lifestyle and thereby inhibits certain chronic diseases and accidental injury which are life shortening (Batty et al. 2007; Gottfredson and Deary 2008). A large epidemiological study in Scotland found that intelligence tests in childhood predict adult morbidity and mortality (Gottfredson and Deary 2008). Lifestyle changes were able to modify mortality in a 5 year study of 7,989 healthy men aged 65–83 years (Spencer et al. 2005). Those men having at least 5 healthy-lifestyle behaviors out of 8 had a lower risk of death. The 8 lifestyle behaviors were not smoking, having no more than 2 alcoholic drinks daily, 3 h physical activity per week, eating meat less than 5 times per week, no salt added to food, body mass index of 25 or less, drinking reduced fat or skim milk (Spencer et al. 2005).

A number of studies have related lifestyle to mortality. The Seneca study linked diet and lifestyle factors to mortality in 2,580 elderly Europeans over 10 years (Haveman-Nies et al. 2003; de Groot et al. 2004). In 70–90 year old men and women adherence to a Mediterranean diet and healthy lifestyle was associated with a 50% lower all-cause mortality (Haveman-Niess et al. 2002; Knoops et al. 2004). Another healthy lifestyle that reduces the risk of chronic diseases lists not smoking, BMI of <25, more than 30 min/day moderate activity, modest alcohol consumption and 40% healthy diet score (Chiuve et al. 2008). From the Framingham Study Goldberg et al. (1996) found that in 50 year old healthy men and women those who survive to 75 years smoked fewer cigarettes per day, had lower systolic blood pressure and higher forced vital capacity.

The largest and longest continuing study of lifestyle and aging is the Nurses Health Study in USA where 77,782 women aged 34–59 and free of cardiovascular disease and cancer in 1980 were followed over 24 years and showed that mortality in 55% could be attributed to smoking, being overweight, lack of physical activity and poor diet (van Dam et al. 2008). It was estimated that healthy living could add 10 years to life. Figures for the PAR, the population attributable rise, showed that

Table 2.2 Effect of healthy lifestyle factors on mortality in middle-aged women showing the population attributal rises (PAR) (from Van Dam et al. 2008)

Variable	Women in high risk category (%)	Death from any cause	
		Relative risk	Population attributable risk (%)
Ever vs. never smoking	56	1.66 (1.59–1.74)	27.9 (24.6–31.2)
BMI $\geq$ 25 vs. 18.5–24.9	48	1.32 (1.27–1.38)	14.2 (11.6–16.9)
Physical activity < 30 min/day vs. $\geq$ 30 min/day	75	1.25 (1.17–1.32)	16.5 (11.7–21.1)
Health diet score in lower three fifths vs. upper two fifths	59	1.25 (1.19–1.30)	12.9 (9.6–16.2)
Heavier drinking or abstaining vs. light to moderate alcohol intake	34	1.18 (1.13–1.24)	7.4 (4.6–10.2)

28% of deaths could be attributed to smoking, 14% to overweight, 17% to lack of physical activity, 13% to unhealthy diet and 7% to alcohol consumption. Thus efforts to eradicate cigarette smoking, to stimulate physical activity and promote a healthy diet should be increased (van Dam et al. 2008) (Table 2.2).

An 11 year prospective study of 20,244 men and women aged 45–79 years, with no known cardiovascular disease or cancer, related four health behaviors to mortality (Khaw et al. 2008). These were: smoking, physical inactivity, moderate alcohol intake, and no fruit and vegetable intake. The study showed that a person with a health behavior score of zero has the same risk of dying as a person with a score of four who is 14 years older (Khaw et al. 2008). In a 26-year follow-up of 1,658 white men (born 1919–1934) in the Helsinki Businessmen Study participants who never smoked lived 10 years longer than heavy smokers (>20 cigarettes per day) (Strandberg et al. 2008). A prospective cohort study of 2,357 healthy men of mean age 72 years within the Physicians Health Study (1981–2006) showed that the probability of a 90 year lifespan was 54% in the absence of smoking, diabetes, obesity, hypertension or sedentary lifestyle (Yates et al. 2008).

With a healthy lifestyle it is possible to increase the survival of men with no history of cardiovascular disease in old age (Spencer et al. 2005). Such a lifestyle includes not smoking, drinking alcohol in moderation, exercising regularly, maintaining normal body weight and eating a high fruit and vegetable Mediterranean-type diet (Haveman-Nies et al. 2002; Spencer et al. 2005). Good self-rated health plus a healthy lifestyle of not smoking and doing more physical exercise are important predictors of survival in elderly women (Ford et al. 2008).

2.7 Life Expectancy in the Twentieth Century

In the twentieth century human life expectancy at birth in 18 developed countries increased spectacularly from 50.3 years in 1900 to 78.3 years in 2000, a gain of 28 years (Tiffen and Gittens 2004). This life extension occurred despite an increase of nearly 10% in calorie intake in 18 developed countries from 1961 to 2000; the calorie increase in United States was 30% (Tiffen and Gittens 2004). High calorie intakes are known risk factors for diabetes and cardiovascular disease, major causes of mortality in later years (Bray 2004). Obviously, factors other than calorie intake are determining survival (Wilmoth 2000; Everitt and Le Couteur 2007). For example, in the first half of the twentieth century control of infectious diseases by better sanitation and nutrition reduced infant and maternal mortality (De Flora et al. 2005). Infant mortality in the first year of life in Australia decreased from 83 deaths per 1,000 live births in 1900 to 5.2 deaths in 2000 (Tiffin and Gittens 2004). Antibiotics and vaccines developed later reduced mortality from infectious diseases (Hinman 1990; De Flora et al. 2005). Motor vehicle accident deaths have been reduced by better design of cars and roads and by legislation to reduce speed on the roads and decrease alcohol consumption (Pless 2004; Cummings et al. 2006).

Since 1970 reductions in death rates in the elderly, largely due to successful medical interventions, have become the main factor increasing longevity (Wilmoth 2000). Mortality in middle and old age from cardiovascular disease has been decreased by drugs and diets to lower blood pressure and cholesterol (Kannel and Thom 1984; Stamler 1985; Sytkowski et al. 1990; Hetzel 2001; Sesso et al. 2003; Everitt et al. 2005; Lloyd-Jones et al. 2006). Coronary artery bypass surgery and implanting defibrillators in the elderly are beneficial (Kaufman et al. 2006; Glock et al. 1996). The gains in life expectancy from preventive interventions in populations at risk may range from 1 month to 5 years or more (Wright and Weinstein 1998). A decline in cigarette smoking has reduced mortality from lung cancer (Kannel and Thom 1984; Jacobs et al. 1999; Wilmoth 2000; Hetzel 2001). These reductions in mortality in old age have led to increases in the numbers of centenarians, which have been doubling every 10 years since 1970 in developed countries (Shibata and Haga 1992; Vaupel 1997; McCormack 2000; Vaupel and Kistowski 2005). There is evidence that the maximum lifespan has increased in Sweden from 101 years in the 1860s to about 108 years in the 1990s (Wilmoth et al. 2000). The greatest lifespan confirmed by birth certificate is that of the French woman Jeanne Calment (Fig. 2.5) born February 21, 1875 and died August 4, 1997, at age 122 years and 164 days and according to Coles (2004) this maximum lifespan is unlikely to be exceeded without a new form of medical breakthrough.

2.8 Life Expectancy in the Twenty First Century

The average life expectancy is continuing to increase. During the twentieth century, living conditions improved and more resources were invested in body maintenance and repair (Holliday 2004; Westendorp 2006), increasing life expectancy. Vaupel

Fig. 2.5 Jeanne Calment the longest-lived human died on 4 August 1997 in France at age of 122 years 164 days (Reproduced with permission of Soylent Communications)

(2000) believes that survival to 90–100 years will be the destiny of many people alive today. Most independent older Australians are optimistic about their future (Quine et al. 2008). Life expectancy at birth in affluent countries is expected to reach 85–87 years by 2050 (Wilmoth 2000). However, projections made by the United Nations are predicting a rise of 25 years in life expectancy at birth over the next three centuries (Kinsella 2005).

Yet the epidemic of overweight and obesity developing in the twenty first century throughout the world is predicted to reduce human survival (Olshansky et al. 2005; Erdman 2006) by increasing the incidence of cardiovascular disease and diabetes (Lavie et al. 2008). Overweight and obesity in children and adults are continuing to increase in prevalence (Ogden et al. 2006) and in the future there will be an increasing burden of obesity-related illnesses (Bessesen 2008).The prevalence of overweight and obesity in childhood has doubled in Australia in the last decade (Waters and Baur 2003). Unfortunately the targeting of the clinical aspects of childhood obesity has had little success (Maziak et al. 2008).

Obesity at the age of 40 confers a 7 year reduction in life expectancy and obesity in combination with smoking reduces life expectancy by 15 years. Being overweight in adolescence increases subsequent morbidity and mortality (Must et al. 1992).

Obesity causes inflammation, insulin resistance, hypertension, hyperlipidemia, leading to diseases such as type 2 diabetes, cardiovascular disease and some cancers. In the twenty first century a major health problem will be obesity linked to diabetes, which is rising in incidence and is expected to double by 2025 (Erdman 2006). The term diabesity refers to the pandemic of diabetes and obesity (Jones 2006). Diabetes increases the risk of cardiovascular disease, the number one cause of death in developed countries (Erdman 2006).

2.9 Overconsumption, Obesity, and Life Expectancy

Due to increasing food intake and reduced physical exercise the incidence of overweight and obesity has been rising steadily in most countries during the last 20 years (Bray 2004), with more than 60% of the population now classified as overweight or obese in Australia, USA, Canada and the UK. In the USA, the prevalence of obesity alone (BMI>30 kg/m^2) has increased from <15% to almost 30% of the population in the majority of states (Fig. 2.1).

Overweight increases the long-term risk of diabetes and cardiovascular disease (Wannamethee et al. 2005), which are life shortening (Peeters et al. 2003; Bray 2004). Data from the Framingham study show that at age 40 being overweight (body mass index BMI of 25–30) reduces life expectancy by 3 years and being obese (BMI >30) shortens life by 7 years (Peeters et al. 2003). Subjects who are very obese (BMI>45) and aged 20–30 years have greater life-shortening effects of 13 years in men and 7 years in women (Fontaine et al. 2003). Thus the greatest effect of excessive calorie intake in humans is probably a loss of 13 years in life expectancy. A large prospective study over 10 years of 527,265 U.S. men and women aged 50–71 years found the lowest mortality occurred in non-smoking men (Fig. 2.6) and

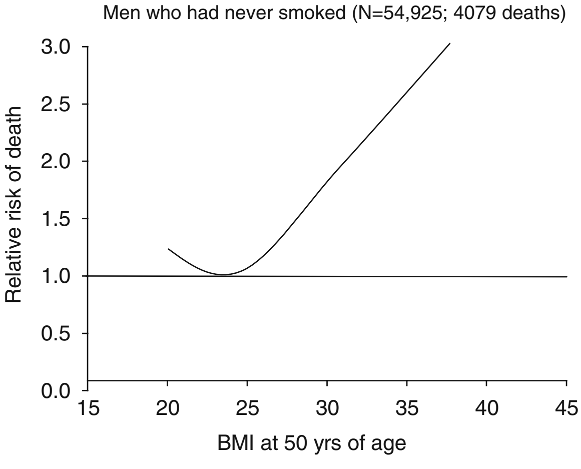

Fig. 2.6 The relative risk of death in men aged 50–71 years is lowest in non-smokers with a BMI of 23.5–24.9 at age 50 years (Adapted from Adams et al. 2006, with permission)

women with a BMI of 23.5–24.9 at age 50 years (Adams et al. 2006); the risk of mortality increased 20–40% if overweight and two to three times if obese.

However, there are studies showing that being overweight in old age has a smaller effect on life expectancy than in earlier life (Elia 2001; Reynolds et al. 2005; Diehr et al. 2008). While obesity and being underweight are associated with excess deaths, overweight has only a small effect (Flegal et al. 2005).

The effects of 6 months 25% CR in overweight human subjects (BMI 25–30) of ages less than 50 years revealed a 10% fall in body weight, plus significant declines in fat mass, fasting insulin level and core body temperature (Heilbronn et al. 2006). Thus 25% CR in overweight subjects was able to reverse two biomarkers of longevity, which ultimately should increase lifespan. Reducing body weight by decreasing food intake and increasing physical exercise (Stubbs and Lee 2004) decreases the risk of developing life-shortening diseases such as diabetes, hypertension, coronary heart disease and various cancers (Bray 2004). There is little doubt that failure to control the obesity and diabetes epidemics in the twenty first century will cause life expectancy to fall (Olshansky et al. 2005).

The problem of overweight has its beginnings in childhood due to increased food intake and less physical exercise (Maziak et al. 2008). It is believed that sugar-sweetened soft drinks may also contribute to the epidemics of obesity and diabetes (Apovian 2004). The rapid rise in obesity among children over the past few decades may result in shorter lifespans of these children relative to their parents (Goran et al. 2003), because overweight and obesity in childhood influences the development of diabetes (Jones 2008) and cardiovascular disease (Baker et al. 2007). Risk factors for coronary heart disease can be identified in overweight children (Baker et al. 2007). Obesity during adolescence in men increases all cause mortality and mortality from cardiovascular disease and diabetes (Dietz 1998). A 26 year follow-up in the Framingham study showed that obesity is an independent risk factor for cardiovascular mortality (Hubert et al. 1983).

In the 1930s Lord Boyd Orr in Britain conducted the Carnegie Survey of Family Diet and Health. In one study overweight in 2,399 children aged 2–14 years in 1937–1939 was found some 57 years later to be associated with increased cardiovascular mortality in old age (Gunnel et al. 1998). This effect of childhood BMI on adult cardiovascular disease was not confirmed in a Scottish study of 11,106 younger children of mean age 4.9 years (Lawlor et al. 2006). However, a large cohort study of 276,835 Danish schoolchildren born in 1930–1976 found that a higher BMI in childhood was associated with an increased risk of coronary heart disease in adulthood (Baker et al. 2007). High food intake studied over 1 week in 3,834 children in 1937–1939 was associated with increased mortality from cancers some 60 years later in a Boyd Orr cohort study (Frankel et al. 1998). These cancers were not related to smoking.

There are also studies showing that human cancer incidence is related to body growth measures such as height (Albanes et al. 1988), leg length (Gunnell et al. 1996) and body mass index (Kune et al. 1990). One study has found a lower incidence of breast cancer in 7,303 Swedish women hospitalised for anorexia nervosa before age 40 years between 1965 and 1998 (Michels and Ekbom 2004).

A prospective study of more than 900,000 US adults studied over 16 years showed that increased body weight was associated with increased mortality for all cancers combined and for cancers at all multiple sites (Calle et al. 2003).

Several studies indicate that overweight increases the risk of dementia and Alzheimer disease (Pasinetti et al. 2007). In an 18 year follow-up of 392 non-demented Swedish adults aged 70–88 years, significantly higher weight was found in women who developed Alzheimer disease, but not in men (Gustafson et al. 2003). A 27 year study of 10,276 men and women aged 40–45 in 1964–1973 examined in 1994–2003, found that obese people had a 74% increased risk of dementia and overweight people a 35% increase (Whitmer et al. 2005).

Successful prevention or treatment of obesity in the twenty first century has to date made little progress. Prevention of obesity in childhood should emphasize healthy eating of low calorie diets plus regular physical activity (Dehghan et al. 2005; Flynn et al. 2006). Unfortunately there is little evidence-based guidance on successful long-term strategies to prevent or treat obesity (Lawlor and Chaturvedi 2006). Bariatric surgery in pediatric patients reduces body weight, but may have serious complications (Treadwell et al. 2008). However, a 6 year survival study of 1,035 obese patients compared with a control group of 5,746 obese patients well matched for age, gender and duration of follow-up, found that bariatric surgery significantly reduced 5 year mortality by 89% (Christou et al. 2004).

2.10 Genetic Mechanisms of the Longevity-Enhancing Effects of CR in Humans

Although reducing food intake has profoundly beneficial effects on aging and pathology, little is known about the genetic pathways that regulate these changes in humans (Barzilai and Shuldiner 2001; Mair and Dillin 2008). The hereditary influences on longevity are most apparent in the oldest-old adults (Abbott et al. 1978; Hornsby 1991). Studies on siblings of long-living people strongly support the presence of hereditary components of successful aging, and thus of human longevity. Siblings of centenarians are three to four times more likely to survive to the 10th decade of life, compared with siblings of non-centenarians (Perls et al. 1998). The immediate ancestors of Jeanne Calment from France who died at the age of 122 years were 10 times more likely to reach 80 years than the ancestral cohort (Robine and Allard 1998).

Rodent studies have shown that complete or partial loss-of-function in genes encoding components of the insulin/IGFI pathway extend life span and the majority of potential longevity genes identified in humans to date also involve the insulin/IGF1 pathway. Genetic polymorphisms in the human IGF1R that result in altered IGF signaling pathway confer an increase in susceptibility to human longevity, suggesting a role of this pathway in modulation of human lifespan (Suh et al. 2008). The klotho protein has also been implicated. In rodents, overexpression of klotho increases lifespan and inhibits the insulin/IGF-1 pathway whereas the knockout animals have reduced longevity (Kurosu et al. 2005). Elderly people homozygous for a gene-spanning haplotype (called KL-VS) that affects klotho gene expression were underrepresented in Bohemian Czechs, Caucasians and African-Americans (Arking et al. 2005). Moreover, a prospective analysis revealed an increased mortality rate for KL-VS homozygous aged individuals (Arking et al. 2005). Another potential longevity gene recently described is the human forkhead box O3A gene (FOXO3A) which encodes an evolutionarily conserved key regulator of the insulin-IGF1 signaling pathway that is known to influence metabolism and lifespan in model organisms. Three polymorphisms in the FOXO3A gene were associated with longevity in long-lived men of Japanese ancestry (Willcox B et al. 2008a) and this was replicated in a population of German centenarians/nonagenarians vs younger controls. Furthermore, the FOXO3A association was considerably stronger in centenarians than in nonagenarians (Flachsbart et al. 2009).

Recent seminal studies in yeast, flies, worms and mice have shown that many aspects of CR are mediated by the silent information regulators or sirtuins (SIR2 in yeast, and SIRT1 in mammals) (Baur et al. 2006: Chen and Guarente 2007). These genes are pivotal regulators of cell survival and longevity, probably through their regulation of mitochondrial biogenesis (Sinclair 2005; Guarente 2005). Currently, there is a search for dietary activators of the sirtuins, which mimic the actions of CR (Allard et al. 2009). One such CR mimetic is resveratrol, a plant polyphenol which by increasing sirtuin activity extends life in several species (Howitz et al. 2003; Baur et al. 2006). To date SIRT1 sequence variation is not associated with exceptional human longevity (Flachsbart et al. 2006).

2.11 Control of Food Intake

If food intake is a major determinant of aging, factors that regulate feeding must play an important role in aging. There is a perpetual desire to eat, which is inhibited by the presence of food in the digestive tract and nutrients in blood. Eating relieves the body's continual state of hunger. Ghrelin synthesized by the stomach stimulates food intake, the secretion of growth hormone and signals the hypothalamic feeding centres (Eisenstein and Greenberg 2003). A second hormone leptin, secreted by fat cells, mediates long-term regulation of energy balance by inhibiting food intake and thereby reducing body weight (Klok et al. 2007). Obese patients are leptin-resistant. Insulin and leptin are said to be involved in long-term maintenance of fat stores (Carrascosa et al. 2009). Leptin is believed to regulate food intake and obesity by interacting with the hypothalamic- pituitary-adrenal axis (Mastorakos and Zapanti 2004) and by this means may have an antiaging action (Shimokawa and Higami 1999).

2.12 Conclusion

Life expectancy at birth in developed countries is rising at the rate of 2 months in each year and is expected to increase as progress in medical science continues to reduce mortality from the major killer diseases. However, overeating and reduced physical exercise resulting in obesity, reduce life expectancy by up to 10 years and may eventually diminish the gains made by medical science. The rising food intake over the last 40 years has led to the epidemics of obesity and type 2 diabetes which are life shortening.

A recommended life style for a long and healthy life should be started in childhood by eating low-calorie foods, such as fruit and vegetables plus whole grain foods and fish, performing regular physical exercise, maintaining a normal body mass index, not smoking or taking illicit drugs and having regular medical check-ups. A healthy lifestyle with a normal body mass index and good medical care over 20 years may increase life expectancy by up to 10 years. Successful treatment of obesity by gastric surgery may add some years to life. Such procedures would extend life by less than the 28 years gained by the successful clinical and dietary interventions in the twentieth century. Food restriction is not recommended in old age.

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Chapter 3
Okinawa: A Naturally Calorie Restricted Population

**Matthew W. Rosenbaum, Bradley J. Willcox, D. Craig Willcox,
and Makoto Suzuki**

3.1 Introduction

Although caloric restriction (CR) is the only non-genetic intervention which has been shown to consistently increase both average and maximum life span of a variety of species, there is still doubt about its efficacy in humans (Lane et al. 2004; Phelan and Rose 2005). Long term studies of humans are exceedingly challenging and certain outcomes (e.g. lifespan) are not tenable for study. This has made the Okinawans a population of special interest for the study of CR. The traditional diet of Okinawans, which is low in caloric density yet high in nutrient density, is purported to be one of the major contributing factors to their longevity and past work has suggested a role for CR in this longevity phenomenon (Hokama 1967; Kagawa 1978; Chan et al. 1999; Willcox et al. 2007a; Akisaka et al. 1996). We review this evidence in the current manuscript.

3.2 The Traditional Okinawan Diet

Although it is well known that the Japanese are the world's longest lived people, less well known is that there is a Northeast to Southwest gradient in longevity, whereby the longest lived of the Japanese are those that inhabit the southernmost islands, known as the Ryukyu Islands (or Okinawa prefecture) (Willcox et al. 2008). The inhabitants of Okinawa prefecture have the longest life expectancy within Japan (and likely the world) at mid-life, in part due to very low risk for age associated diseases such as cancer, coronary heart disease, stroke and diabetes (Willcox et al. 2007a).

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Much of the longevity advantage in Okinawa is thought to be related to lifestyle factors, such as the traditional Okinawan diet, which is low in calories yet nutritionally dense, particularly with regard to phytonutrients such as flavonoids and other antioxidants (Willcox et al. 2004).

The traditional dietary (food) pattern in Okinawa has the following characteristics:

1. Low caloric intake;
2. High consumption of vegetables;
3. High consumption of legumes (mostly soy in origin);
4. Low consumption of dairy products;
5. Low consumption of meat and meat products;
6. Moderate consumption of fish products (especially in coastal areas);
7. Low consumption of refined starches and low overall Glycemic Load (GL);
8. Low consumption of saturated fat; high monounsaturated to saturated fat ratio; high consumption of omega-3 fat;
9. Moderate alcohol consumption.

Several of the above components of the traditional Okinawan diet are shared with other healthy dietary patterns such as the traditional Mediterranean diet or DASH (Dietary Approaches to Stop Hypertension) diet (Appel 2008). A comparison of the nutrient profiles of the three dietary patterns shows that the traditional Okinawan diet is the lowest in calories and fat, particularly in terms of saturated fat (Willcox et al. 2010, in press). The traditional Okinawan diet is also highest in carbohydrates, in keeping with the very high intake of anti-oxidant rich yet calorie poor root vegetables, particularly the dietary staple – sweet potatoes – as well as a myriad of other vegetables.

Not surprisingly, all three patterns have been found to be cardio-protective, in part due to healthy fat intake (low intake of saturated fat and high intake of omega-3 and monounsaturated fat) (Appel 2008). However, other mechanisms such as high vegetable and legume consumption, low GL, high flavonoid and other antioxidant consumption, are also likely contributing to lower risk for cardiovascular diseases, some cancers and other chronic age associated diseases (Willcox et al. 2007b). Since no dietary factor has exceeded the potential role of CR for enhancing healthy aging in animal studies it is this factor that we will explore further in the current paper.

3.3 Early Studies on Energy Intake in Okinawa

Some of the earliest reported data on energy intake in Okinawa comes from Hokama et al. (1967), who reported that Okinawan school children consumed only 62% of the calories of mainland Japanese school children in the early 1960s. Kagawa (1978) reported that caloric intake for adults in Okinawa was only 83% of the Japanese average and presented anthropometric and medical data from small samples of older Okinawans which were consistent with the phenotypic effects of CR (short stature, low BMI, minimal body fat). Similar data were presented by Akisaka et al. (1996) and Chan et al. (1999), who reported dietary and phenotypic data in older Okinawans which were consistent with CR. However, these studies did not take into account the dramatic change in the Okinawan diet and lifestyle which occurred from the 1950s to present. Nor do these reports adjust energy requirements for the smaller body size of Okinawans compared to mainland Japanese. A smaller body size would result in a lower energy requirement and could lead to a false perception of CR where none exists. In order to judge whether or not elderly Okinawans were truly undergoing a form of natural CR during much of their lives, and if so, the actual effects on their physiology, a different approach is necessary.

3.4 The Traditional Okinawan Diet and CR

In order to effectively assess lifelong energy balance (and thus potential CR status) of older Okinawans, who enjoy remarkable health and longevity advantages, it is necessary to assess energy balance from a life course perspective. Willcox et al. (2007a) took this approach when they studied archived dietary and anthropometric data from the U.S. Office of the Civil Administrator of the Ryukyu Islands (Okinawa) and Okinawa Prefectural Government. Population data (diet, anthropometry) for Okinawan adults was assessed at approximately 10 year intervals from 1949 to 1998. These records were also utilized to assess energy balance for Okinawan septuagenarians when they were young and middle-aged adults (Willcox et al. 2007a). Most assessments used 3-day food records of usual dietary intake along with dietician interviews to estimate caloric intake. Anthropometric measurements (height and weight) and demographic data (age, occupation), were used to estimate energy expenditure using the Harris-Benedict equation (Frankenfield et al. 1998). Energy balance was estimated by subtracting the estimated energy expenditure from estimated caloric intake.

From these data we can see that Okinawans appeared to be in an approximately 11% “energy deficit” from 1949, when data first became available, until the 1960s (Fig. 3.1). Since the traditional diet had not changed prior to the 1950s we can surmise that the “energy-poor” environment was a long term pattern at least until that time. Physiological adaptations consistent with living in a state of long-term energy deficit should include a low BMI. In support of this, the average BMI of adult Okinawans remained stable at a lean 21 kg/m^2 until the early 1960s, during which Okinawan adult BMI began to rise, coincident with a measurable shift to a positive energy balance.

To quantify the level of CR in the Okinawan population in 1949, when current septuagenarians were young adults, Willcox et al. (2007a) assessed population data. These data suggest an energy intake of about 1,785 kcal/day compared to an estimated energy requirement of 2,003 kcal/day, calculated from the Harris-Benedict equation. While these are rough estimates, they do support an energy deficit of approximately 218 kcal/day (10.9%) per established physicologic norms.

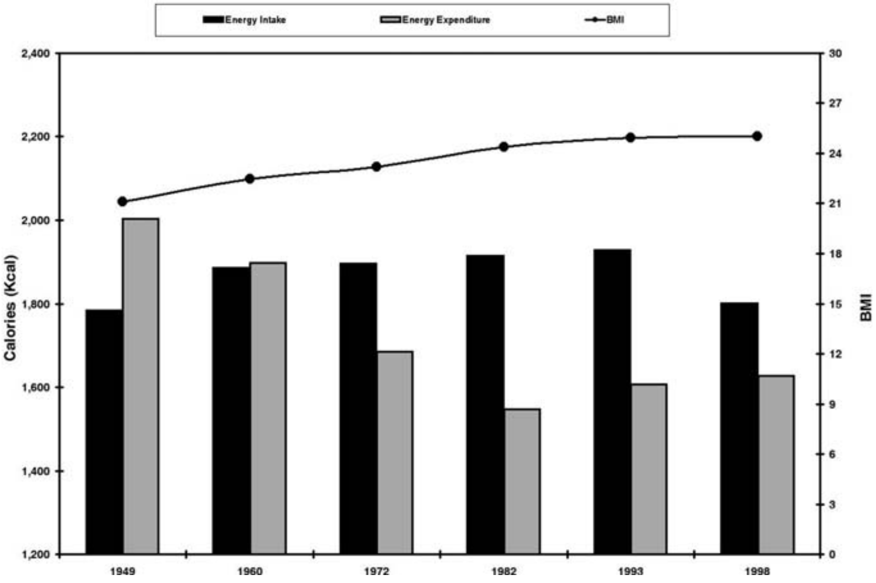

Fig. 3.1 The BMI, energy expenditure and energy intake of Okinawans between 1949 and 1998

This argues for a relative, naturally occurring “caloric restriction” in the range of 10–11%. In animal models this would be considered mild but capable of inducing measurable phenotypic effects, including anthropometric effects, health effects and lifespan advantages. The average BMI of 21.2 for the Okinawan adult population at that time is quite lean and is consistent with an adaptive response to CR. This is anthropometric evidence of the presence of a CR phenotype.

Conversely, by 1972, calculations using adult population data in Okinawa demonstrate a positive energy balance of 212 kcal/day. This represents a positive energy shift of over 400 kcal/day. By this time, a higher BMI of 23.3 is now apparent, suggesting that Okinawan adults ceased to be in a CR state between the 1960s and 1970s. For comparative purposes we juxtapose these findings with the U.S. population who would not be expected to have been in a “negative energy balance” nor have had a CR phenotype. As expected, energy balance and BMI were comparatively higher in American subjects from the same birth cohort (data from the NHANES I study; collected from 1971 to 1974, National Center for Health Statistics 1978). These data demonstrated a positive energy balance of 239 kcal/day and an average BMI of 25.6 for similarly aged Americans.

Since a key requirement for CR is a relative energy deficiency without malnutrition, the traditional Okinawan diet was analysed in order to determine whether or not widespread nutritional deficiencies were occurring and to assess the presence of other potential health-enhancing food components. Notable trends were a high intake of the antioxidant vitamins C, E, as well as folate and B_6, which were 289, 190, 295 and 221% of the Japanese Recommended Dietary Allowance (RDA)

Table 3.1 Micronutrient sufficiency of the traditional Okinawan diet and Japanese diet

	Amount	RDAJ[1] (%)	Amount	RDAJ[1] (%)
Micronutrient	Okinawa, 1949[2]		Japan, 1950[3]	
Vitamin A (RE)	602	110	337	62
Vitamin E (mg)	16.6	190	6.3	72
Vitamin K (mcg)	87.6	160	65.8	120
Vitamin B_1: thiamin (mg)	1.4	137	1.1	113
Vitamin B_2: riboflavin (mg)	0.5	45	0.5	47
Niacin (mg)	13.2	93	18.1	127
Vitamin B_6: pyridoxine (mg)	3.0	221	1.6	118
Folate (mcg)	557.4	295	267.2	141
Vitamin B_{12}: cobalamin (mcg)[4]	0.6	27	4.0	176
Vitamin C (mg)	273.4	289	94.9	100
Calcium (mg)[5]	505.3	82	325.5	53
Iron (mg)	11.6	109	11.0	103
Phosphorus (mg)	864.1	115	1191.2	159
Magnesium (mg)	396.1	151	327.4	125
Potassium (mg)	5199.6	272	2712.3	142
Zinc (mg)	6.2	62	10.6	107
Sodium (mg)	1133.0	113	2450.8	245

[1] Micronutrient requirements are from 6th Recommended Dietary Allowances for the Japanese (RDAJ) 1996.

[2] Calculated from U.S. National Archive, archived food records, 1949.

[3] Calculated from Japan National Nutrition Survey, 1950.

[4] Periodic festivals (approximately monthly) in which pork and other meats were consumed are not accounted for in this analysis.

[5] Okinawan drinking water is high in calcium and other minerals which are not accounted for in this analysis.

(Table 3.1). However, intake of vitamins B_2 and B_{12} were relatively low at 45 and 27% of RDA respectively. As reported in Willcox et al. (2007a) there was physical evidence for some mild nutritional deficiencies of these two vitamins in a minority of the population but widespread and severe nutritional deficiencies were not apparent.

If CR was present in Okinawans there should also be evidence on medical examination. That is, physical characteristics and physiological assessment should be consistent with CR. Kagawa (1978) and Chan et al. (1999) reported anthropometric differences in Okinawans versus other Japanese, particularly shorter height and lower body weight in Okinawans, consistent with CR. Todoriki et al. (2004) reported well documented evidence for lower body weight and shorter stature in thousands of Okinawan school children over a several decade period versus other Japanese, also consistent with CR. Furthermore, medical examination of adult women in 1949 revealed a notable finding that is consistent with CR. That is, there was a relatively high prevalence of delayed menstruation in young women and deficient lactation in recent mothers, both potential indicators of low caloric intake and/or low body fat levels in women (Meyer et al. 1990; Dewey 1998).

DHEA, a potential biomarker of aging (Lane et al. 1997) was measured in 54 Okinawan septuagenarians (as reported in Willcox et al. 2007a), who, according to previously mentioned population data, would likely have undergone CR until at least middle age. These data were quite revealing. As expected from the CR hypothesis, mean values of DHEA in male Okinawan septuagenarians were significantly higher than in similarly aged non-CR American men (2.59 ng/mL as compared to 2.00 ng/mL, $p < 0.001$). An even larger difference was seen in similarly aged Okinawan women (3.03 ng/mL) compared with non-CR Americans (1.13 ng/mL, $p < 0.001$; Fig. 3.2).

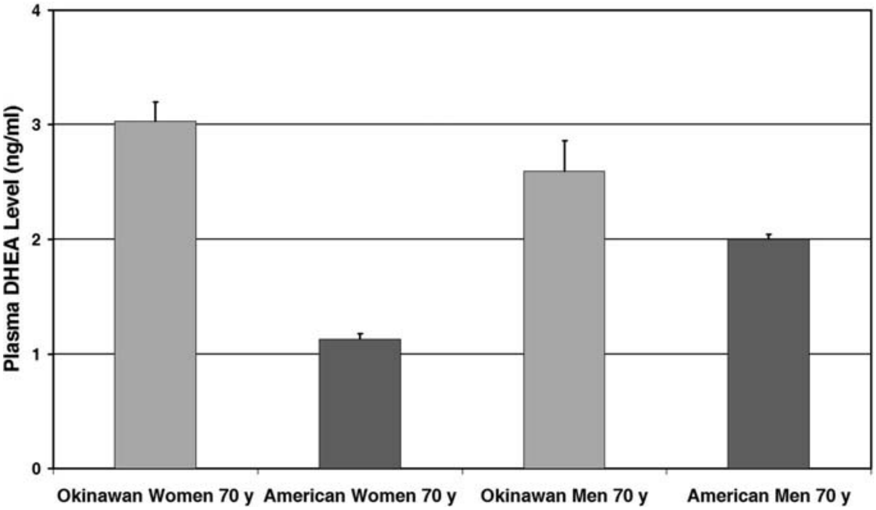

Fig. 3.2 A comparison of plasma DHEA levels in older Okinawans and Americans

In addition, demographic evidence for CR was sought. That is, if CR-induced slower aging was occurring in Okinawans, there should be a rightward shift in the survival curve, as seen in animal studies (Weindruch et al. 1986), with an increase in both median life span (defined as the age of death for the 50th percentile of the population) and maximum life span (defined here as the age of death for the 99th percentile of survival). Figure 3.3 shows the survival curves for the Okinawan population compared to Japanese and American populations for 1995 based on a conservative survival model (LIFEREG Procedure) (Willcox et al. 2007a). Consistent with the effects of CR in animal experiments, there is a distinct rightward shift in the survival curve for Okinawans compared to the Japanese and Americans for both median lifespan (83.8, 82.3 and 78.9 years respectively) and maximum lifespan (104.9 compared to 101.1 and 101.3 years, respectively).

Finally, as CR is thought to greatly decrease the risk for and the onset of age-related diseases (Maeda et al. 1985), it would be expected that Okinawans who underwent CR for a substantial period of time would suffer from lower risk for age-related diseases. This should be reflected in lower disease specific mortality rates. Therefore, we compared Japanese and Americans to age-matched Okinawans.

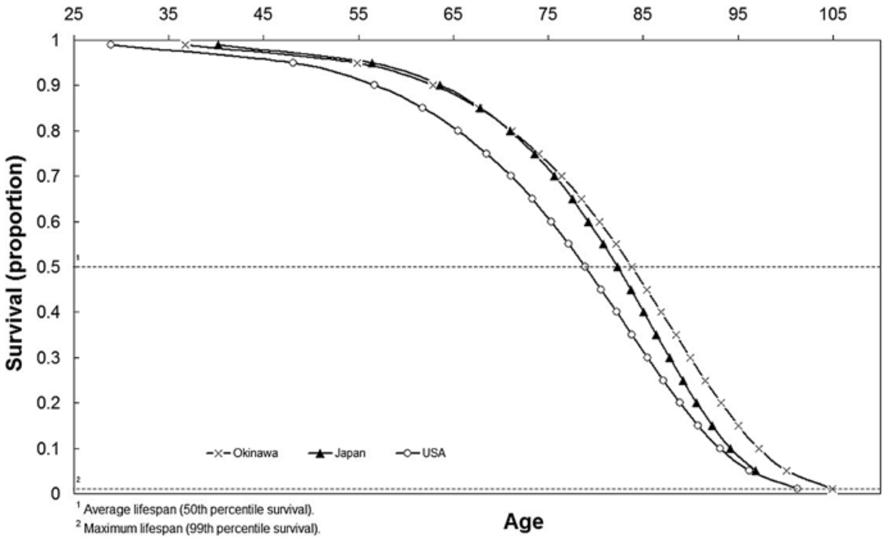

Fig. 3.3 Survival curves for Okinawans compared to other people in Japan and Americans

Indeed, there was substantially lower mortality from coronary heart disease and multiple types of cancers linked to CR (Fig. 3.4). A shift in age-related disease to older ages would be reflected in lower age-adjusted death rates for the whole Okinawan population and likely higher functional status at older ages, which is

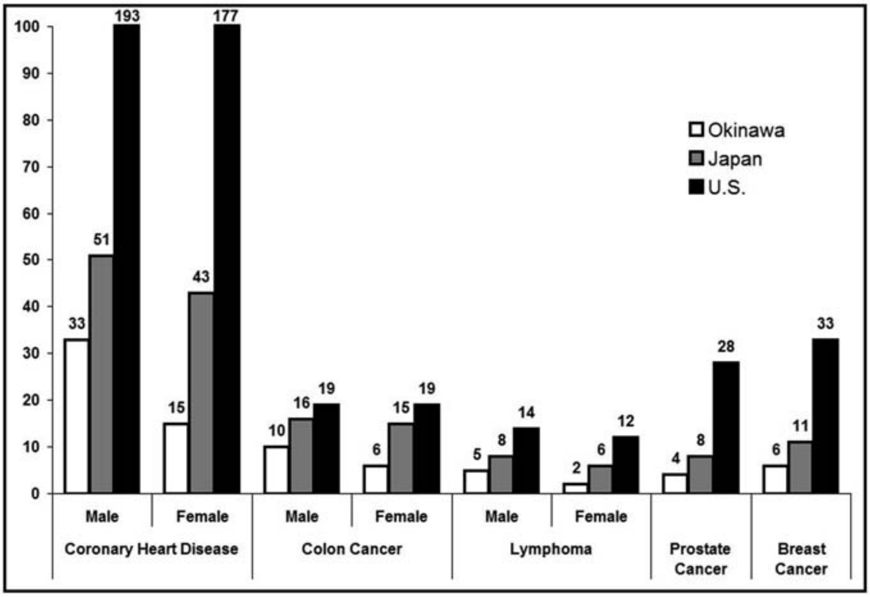

Fig. 3.4 Comparison of Okinawan, Japanese and American mortality rates for coronary heart disease and several cancers

precisely what is observed (Willcox et al. 2007a, b). This is a further indication that age retardation due to CR may have occurred.

3.5 Other Potential Contributors to Longevity

Because Okinawans come from an island population with a relatively restricted gene flow, the possibility of genetic factors being partially responsible for Okinawan longevity, or for some of the phenotypic findings, cannot be ignored. There does appear to be a familial component to longevity for centenarians in Okinawa but further investigation of a population-wide longevity advantage has not yet been reported (Willcox et al. 2006). There also appears to be a genetic component to DHEAS (a serum hormone related to DHEA) levels in humans. Therefore, populations may differ on this basis (Rotter et al. 1985). However, LaCroix et al. (1992) showed that Japanese men actually have lower levels of dehydroepiandrosterone sulfate (DHEAS) than American men at younger ages. Therefore, the differences we see in the Okinawans (a Japanese subpopulation) and Americans appears consistent with a slower decline with aging in Okinawans – again also consistent with CR in animal studies.

Likewise, if CR is partly responsible for the health and longevity advantages of older Okinawans then such advantages should gradually disappear if the CR phenomenon has ended. Indeed, changes in diet and lifestyle that have resulted in positive energy balance in younger and middle-aged Okinawans have markedly reduced their prior longevity advantages. This also provides evidence that the exceptional longevity of the Okinawan elderly is not due solely to genetic factors but may be, in part, due to CR.

On the other hand, nutritional factors aside from CR may be partially responsible for the longevity of Okinawans. Advantages in dietary patterns include healthier fat intake, lower glycemic load, lower salt intake, high plant food consumption, regular sanpin (jasmine) tea drinking, among other healthy habits. Physical activity also appeared to contribute to the relative energy deficit observed in Okinawans until the 1970s. Such health habits have all been associated with reduced risk for morbidity and/or mortality in animals and humans (Willcox et al. 1995; Zimmerman et al. 2003; Jenkins et al. 2004). However, unique among these factors, is the consistent effect of CR on maximum life span, as indicated by a rightward shift of the survival curve (Masoro 1990). Therefore, although other nutritional and other lifestyle factors may be partly responsible for the increased longevity of the Okinawans they cannot account for the increased maximum lifespan of this population (Fig. 3.3).

3.6 The Future of Okinawan Longevity

The traditional Okinawan diet underwent rapid post-war Westernization, most notably in terms of increased fat intake. Rapid Japanization also occurred and resulted in the replacement of the staple carbohydrate, the sweet potato, with energy

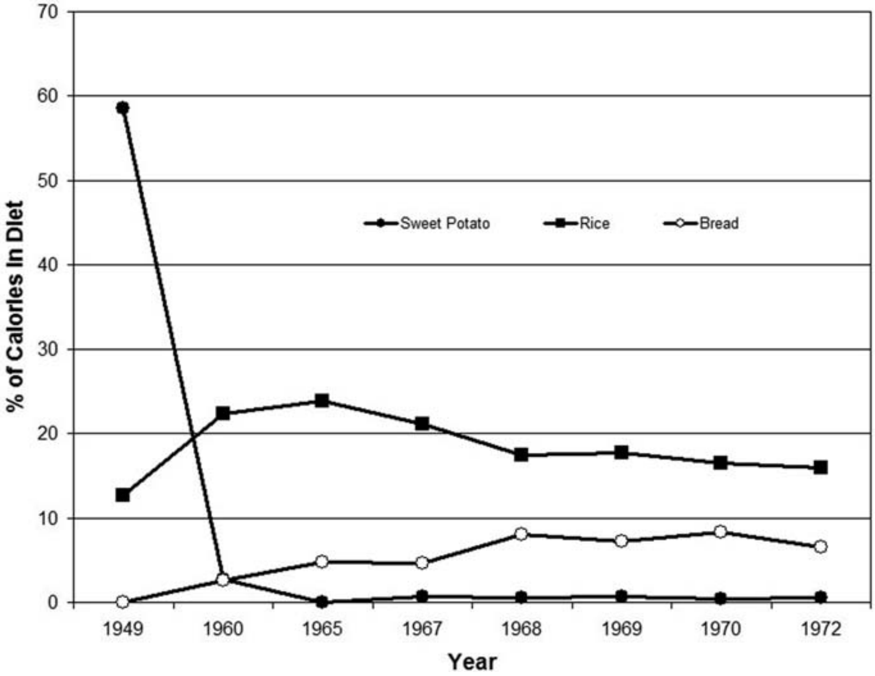

Fig. 3.5 Change in the staple diet of Okinawans between 1949 and 1972

dense white rice and other lower quality, high GI carbohydrate sources (Fig. 3.5). These high energy density foods contributed to an overall increase in caloric intake. A concomitant decrease in energy expenditure also gradually occurred such that by 1972 Okinawan adults were in positive energy balance and the CR phenomenon appeared largely over.

Since the 1970s a further lifestyle shift has gradually taken place with overeating, inactivity, and obesity emerging as major public health challenges (Todoriki et al. 2004). Overconsumption of foods that are calorie-dense, nutritionally-poor, highly processed and rapidly digested can contribute to a cluster of metabolic abnormalities, including central obesity, hypertension, dyslipidemia, glucose intolerance, insulin resistance and inflammation (Tanaka et al. 2005). These metabolic problems (commonly referred to as “metabolic syndrome”) are all independent risk factors for the development of type 2 diabetes, cardiovascular disease, and other lifestyle-related diseases.

As would be expected when faced with such a rapid nutrition transition, younger Okinawans growing up during the post-WWII period have been facing higher risk for morbidity and mortality when compared to other areas of Japan that have been less affected by these large dietary and lifestyle shifts. Indeed, Okinawa currently suffers from the highest obesity rates in Japan and metabolic syndrome has become a significant burden (Tanaka et al. 2005).

Moreover, growth rates in average life expectancy (at birth) have stagnated and there now exists a sharp contrast between the longer living older generations (at lower risk for age-related disease) and the post-nutrition transition generations, who have higher risk for lifestyle related disease than other Japanese and concomitant higher mortality risks (Todoriki et al. 2004).

3.7 Conclusion

Although it is difficult to argue that the health and longevity advantages enjoyed by the current generation of elderly Okinawans are entirely due to the beneficial effects of CR, it appears that these Okinawans underwent a form of naturally occurring, mild CR (approximately 11%) for a large portion of theirs lives. This is consistent with their significantly lower morbidity and mortality risk from a wide range of age-related diseases. The evidence from medical, epidemiologic and demographic sources supports a CR phenotype in these elderly Okinawans and includes: short stature, low BMI, high blood DHEA levels, extended mean and maximum lifespan.

This healthy aging phenomenon lends credence to the argument that there has been a salutary effect of "naturally occurring" CR in Okinawa, with similar health outcomes to those seen in controlled experimental studies employing animal models. Recent publications from long-term interventional studies of primates (Colman et al. 2009) and short-term interventional studies in humans (Redman et al. 2009) are providing additional evidence for the potential efficacy of CR to improve human health and longevity. More research is needed on the effects of CR in human populations.

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Chapter 4
Aging and the Effect of Calorie Restriction in Rhesus Monkeys

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4.1 Overview of CR Studies in Rhesus Macaques

Intramural investigators of the National Institute on Aging (NIA) launched the first study of calorie restriction (CR) in nonhuman primates (NHPs) in 1987 and expanded that initial study with additional monkeys in subsequent years. Investigators at the Wisconsin National Primate Research Center (WNPRC) initiated a similar study in 1989. Male and female rhesus monkeys (*Macaca mulatta*) in both longitudinal studies were randomly grouped into CR or control (CON) diet groups. NIA monkeys ranged in age from one to 23 years when they were assigned to the study and thus data from this site is also affected by age of onset of the treatment. Male monkeys were juvenile (J: 1–2 years), adolescent (A: 3–5 years), and old (O: 16–23 years). Female monkeys were added as three cohorts: juvenile (J: 1–3 years), adult (A: 6–14 years), and old (O: 16–21 years) (Mattison et al. 2005). WNPRC monkeys were all adults (6–14 years) at the onset (Kemnitz et al. 1993; Ramsey et al. 2000). Rhesus monkeys in captivity have an average lifespan of 25 years and the maximum lifespan observed is 40 years (Bodkin et al. 2003).

Both study sites designated a 30% restriction which was instituted gradually over a 3 month period. Because some NIA monkeys were pre-pubertal at the onset of the study, it was necessary to adjust food allotments to allow for growth and development. The objective for the NIA study was to provide sufficient food to the CON group to meet estimated energy requirements based on age and body weight as determined by data from the National Research Council. Thus NIA constructed a body weight-based food allotment chart to guide feeding requirements. All monkeys on the NIA study receive the same food which was modified from a standard National Institutes of Health (NIH) monkey chow formula and enriched with an additional 40% of the vitamin and mineral mix to ensure that the CR monkeys receive 100%

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of the recommended daily allowance. NIA monkeys are fed twice daily and receive a daily novel, low calorie food treat during one of the three daily health checks.

All WNPRC monkeys were initiated as full grown adults therefore each monkey's food intake was monitored for 3 months to determine baseline intake. Similar to NIA, food was reduced by 10% per month for 3 months for the CR group. However, WNPRC monkeys are fed only once per day and the diet formulation for only the CR monkeys is enriched by 30% with additional vitamins and minerals.

Both studies use specially formulated diets of extruded biscuits. NIA uses a natural ingredient diet with 17% protein, 5% fat, and 57% carbohydrate, 6.8% of which is sucrose (Ingram et al. 1990). WNPRC study monkeys are fed a purified diet made of 13% protein, 10.6% fat, and 61% carbohydrate, 44.9% of which is sucrose (Kemnitz et al. 1993).

4.2 Body Composition and Sarcopenia

Body composition is a measure of energy economy reflecting the difference between energy intake and expenditure. Alterations in body composition are directly associated with pathologies, including obesity, osteoporosis, and sarcopenia (the loss of muscle mass and function that occurs with normal aging). In humans, beginning at age 30, approximately 3–8% of muscle mass is lost each decade and this rate of decline accelerates after the age of 60 (Holloszy 2000; Melton et al. 2000), with the loss of vastus lateralis (VL) mass approaching 50% by the eighth decade of life (Lexell et al. 1988). This loss of muscle mass is increasingly recognized as a biomarker of aging, predicting an increased risk for disability and mortality (Melton et al. 2000; Roubenoff and Castaneda 2001; Faulkner et al. 2007). The sarcopenia is believed to be involved in the manifestation of other later-life pathologies including diabetes, osteopenia, and obesity (Dutta 1997) and it is clearly related to increased risk of injury, decreased quality of life, and increased healthcare expenditures. For the year 2000, it was estimated that 1.5% of total health care expenditures in the United States, totaling 18.5 billion dollars, was attributable to sarcopenia (Janssen et al. 2004). Therefore, the economic impact of even a modest reduction in sarcopenia, perhaps through a CR-induced alteration of the aging process, would be appreciable.

Sarcopenia has been previously characterized in aging rodents. For example, in the long-lived Fischer x Brown Norway F_1 hybrid rat strain (mean lifespan 33 months, maximum lifespan 43 months), peak muscle mass occurs between 18- and 21-months of age and then VL and rectus femoris (RF) mass declines approximately 60% and 33–53%, respectively, by 36 months (Wanagat et al. 2001). This loss of muscle mass occurred concomitant with fiber loss, and fiber atrophy accounted for a high proportion of the decline (Wanagat et al. 2001; Bua et al. 2002; McKiernan et al. 2004). However, there are limitations to the rodent aging model which fortunately can be overcome with rhesus monkeys. In addition, the relatively large size of rhesus monkeys, particularly in comparison to other commonly used model species (that is, rodents), makes them particularly useful in sarcopenia investigations where standard human techniques, such as dual-energy x-ray absorptiometry (DXA), can be easily used.

As a first step to develop the rhesus monkey model of sarcopenia, age-related muscle mass loss in laboratory-housed rhesus monkeys was characterized (Colman et al. 2005). Both cross-sectional and longitudinal studies were used to determine when muscle mass loss occurs and the degree of sarcopenia that these monkeys undergo. In the cross-sectional study, DXA assessments of estimated skeletal muscle mass (ESM, sum of the lean mass in the arms and legs) in a large group of males across the lifespan indicated that they had approximately 4.5 kg of muscle at 10–14 years and continued to gain muscle mass between 14 and 19 years. After 20 years of age there were significant decreases in ESM. Specifically, there was an approximately 18% loss of ESM between 15–19 and 20–24 years and an additional 12% loss by $\geq$25 years (average lifespan =27 years) for a total muscle mass loss of 26.7%. When examined longitudinally, there was a similar pattern of muscle gain and loss. Males reached their maximum total body lean mass at 15.6±2.7 years of age and maximum ESM at 15.8±2.5 years of age. A 20% loss in muscle mass was seen by 23.2±3.3 years of age. Female rhesus monkeys reach peak total body lean mass and muscle mass earlier (14.8±2.6 years, 14.1±2.8 years; respectively) than males, and they had a more subtle change in muscle mass over time so that significant change in ESM was not observed until $\geq$25 years of age. However, the longitudinal study indicates a 20% loss in muscle mass by 24.5±6.1 years of age, only slightly later than observed in the males.

WNPRC recently assessed the ability of CR to prevent, or delay, the onset of sarcopenia in 30 adult male CR (n=15) or CON (n=15) monkeys that had been on the study for 11 years (years 6–17 following study initiation) (Colman et al. 2008). Through year 9 of study, both diet groups had approximately the same amount of body-weight adjusted ESM. But by year 10, all monkeys began to show a decline in adjusted ESM; however, the decline was much more rapid for the CON monkeys. That is, the CR monkeys retained more adjusted ESM as they aged. From year 14 to the end of the analysis period, the CR monkeys had significantly higher levels of adjusted ESM than the CONs. These data support the theory that age-related loss of muscle mass, or sarcopenia, is delayed and possibly prevented, in WNPRC's rhesus monkey model of adult-onset, moderate CR.

4.3 Female Reproduction Studies

As females age, numerous hormonal changes along with a declining number of follicles in the ovary lead to menopause, or the complete cessation of spontaneous menstrual cyclicity. Today, more women are choosing to delay reproduction for career and financial reasons. Because of this shift, an emphasis has been placed on extending female fertility and subsequently delaying menopause in order to broaden the window of opportunity for a healthy conception later in life. In extending lifespan and healthspan, CR may also extend reproductive years.

Primates are unique in that they are the only mammals that menstruate. Rhesus monkey menstrual cycle mirrors the hormonal profile of human females at 24–34 days (Gilardi et al. 1997). With advancing age, there is a similar decrease in serum estradiol and, like women, aging rhesus monkeys show increased follicle stimulating hormone (FSH) levels along with decreased Inhibin B (INHB) as well as amenorrhea, the hallmark sign of true menopause (Downs and Urbanski 2006). Older animals also exhibit ovarian attrition and evidence of follicular reservoir depletion, having scattered and atretic follicles, few primordial follicles and reduced amounts of stromal tissue (Nichols et al. 2005), along with a decreased total number of regular menstrual cycles and percentage of normal length cycles (Lane et al. 2001). Preliminary data from NIA and the Oregon National Primate Research Center (ONPRC) have supported this finding.

Research over the last decade of both long- and short-term CR has helped elucidate the role of calories and energy balance on reproductive function and aging. Initial observations from the NIA study of premenopausal female rhesus monkeys indicated no disruption to menstrual cycling or reproductive hormone levels between CON and CR monkeys (Lane et al. 2001). Follow-up observations of pre-, peri- and post-menopausal monkeys were consistent with the earlier reports. The number of monkeys displaying irregular menstrual cycles increased with age without a diet effect. Similarly, no disruption of age-related increases in FSH or decreases in INHB were observed, which are emerging as reliable predictive markers in the menopausal transition. Continued observation of these animals as they progress toward menopause will address the question as to whether or not CR remains a protective mechanism against reproductive senescence.

In contrast to the findings in the long-term CR colony, a study focused on developing a model of nutritional amenorrhea reported that normal weight, 6–10 year old rhesus monkeys ceased ovulation following a 23% reduction in calories in a relatively short time span (62±13 days) (Lujan et al. 2006a). Energy stores appeared to play a role in the effect since the one obese monkey in this study did not respond with anovulation until much later and it required a 42% drop in weight. The disparity in anovulation effect between these two CR paradigms may be due in part to total energy intake. At an approximate 30% reduction, NIA monkeys were getting 1,709±55 kJ compared to 1,504 ± 148 kJ for the Lujan et al. study and an approximate restriction of 21%. Additionally, data from NIA monkeys reflect long-term CR (>6 years) while the CR induced anovulation reported by Lujan et al. was an immediate response to a decrease in calories.

In this acute model of CR, Lujan et al. (2005) challenged ovariectomized monkeys with estrogen/progesterone to induce a gonadotropin surge. Compared to their baseline response at a normal body mass index, when restricted, the positive feedback loop was disrupted and inhibited gonadotropin release indicating that a short-term CR paradigm can alter the hypothalamic-pituitary response. Lujan et al. (2006b) have ruled out the role of leptin in this effect. When re-infused to CR monkeys for 16 weeks, leptin did not trigger FSH release or ovulation.

Based on observations from both acute and long-term CR studies, many factors appear to contribute to the hormonal and menses responses to a calorie deficit. Clearly, an acute nutritional deprivation can cause a hormonal dysregulation that disrupts the reproductive cycle. However, as observed following a long-term CR protocol, monkeys have adaptive mechanisms that compensate or adjust to the reduced number of calories and create a new set point.

4.4 Immune Function and Immune Senescence

Aging of the immune system results in a diminished immune response to infection and vaccination. This state of diminished immune function is commonly referred to as immune senescence. Data from several studies in rodents strongly suggest that CR can revitalize the immune system (Masoro 2005) by delaying the onset and/or reducing the severity of immune senescence. Clinical studies have defined several hallmarks of immune senescence that have been collectively referred as the “immune risk phenotype” (IRP, Table 4.1) (Pawelec et al. 1995).

Table 4.1 Changes in immune system associated with the immune risk phenotype

Increased	Decreased
Effector memory (CD28^{neg}) CD8 T cells	Naïve T cell frequency
Memory T cells expressing NK cell markers	Thymic production of naïve T cells
T cell clonal expansions	T cell repertoire diversity
B cell clonal expansions	T cell proliferation
CD5^{+} autoantibody-producing B cells	CD4:CD8 T cell ratio
Frequency of cytomegalovirus-specific T cells	B cell frequency
IL-6	B cell repertoire diversity
TNF-a	Response to vaccination
Cytotoxic natural killer cells	Telomerase activity

The most striking hallmark of the IRP is the dramatic loss of naïve T cells (Wikby et al. 1998; Fagnoni et al. 2000). This loss is largely due to diminished thymic output (Naylor et al. 2005), and is further compounded by life-long exposure to pathogens that results in significant conversion of naïve T cells to memory T cells (Czesnikiewicz-Guzik et al. 2008). The loss of naïve T cells is accompanied by the accumulation of memory T cells, especially terminally differentiated memory CD8 T cells that have lost CD28 expression (Vallejo 2005). This shift towards memory T cells results in a reduced diversity of the T cell repertoire, which is further exacerbated by the appearance of T cell clonal expansions (TCE) (Posnett et al. 1994; Schwab et al. 1997). Another hallmark of immune senescence is the loss of T cell proliferative capacity (Miller 1996). Aging of the immune system is accompanied by an increase in the production of inflammatory cytokines, especially IL-6 and TNF-α (De Martinis et al. 2005; Wikby et al. 2006). This phenomenon, often referred to as inflammaging, is believed to contribute to the development of several chronic diseases such as Alzheimer’s and osteoporosis (Ginaldi et al. 2005). The IRP can be used to predict susceptibility to several age-related chronic and infectious diseases (Wagner et al. 2004) as well as longevity (Strindhall et al. 2007; Larbi et al. 2008).

Studies as early as 1973 showed that life-long CR significantly increased mitogen-induced T cell proliferation in aged mice (Walford et al. 1973). Since decreased T cell proliferation is a hallmark of immune senescence, these early observations strongly suggested that CR might delay aging of the immune system. Indeed, follow-up studies confirmed that CR maintained T cell proliferative capacity in aged mice and rats (Grossmann et al. 1990), most likely by preventing the accumulation of non-replicative, non-functional T cells through enhanced apoptosis of senescent T cells (Spaulding et al. 1997a). Further, CR initiated at weaning and maintained throughout the entire lifespan promoted the retention of circulating naïve CD4 and CD8 T cells by delaying the rate of thymic aging (Chen et al. 1998). Further studies elucidated that CR preserves hematopoetic stem cell (HSC) progenitor numbers in the bone marrow and their ability to repopulate hosts to levels that were even higher than those achieved with HSC from young animals (Chen et al. 2003). Moreover, CR reduced the levels of circulating inflammatory cytokines IL-6 and TNFα in aged mice (Effros et al. 1991a; Spaulding et al. 1997b), which in turn resulted in decreased incidence of autoimmune disease (Jolly and Fernandes 1999; Jolly 2004) and cancer (Weindruch and Walford 1982; Volk et al. 1994). The impact of CR on the response to infection remains controversial. Earlier studies showed that CR improved the T cell response to influenza (Effros et al. 1991b). However, more recent studies showed that CR mice were more susceptible to mortality following influenza or bacterial challenge than CON animals (Gardner 2005; Kristan 2007). One confounding factor is that infection with the pathogens studied in these reports resulted in significant weight loss, which could explain the increased susceptibility of CR animals.

Although specific pathogen free (SPF) laboratory rodents have been invaluable in the characterization of the impact of CR on the immune system, the inbred nature of SPF rodents, their short lifespan, and the scarcity of murine homologues of human pathogens restrict the successful transfer of immunological discoveries made in murine models to the clinical setting (Hein and Griebel 2003). NHP models offer a unique opportunity to carry out mechanistic studies in a species that closely mimics human biology but yet can be maintained under tight laboratory conditions. The impact of CR on immune function in long-lived primates remained poorly understood for several years until immunological studies were initiated using the both the NIA (Lane et al. 1992; Roth et al. 1999) and WNPRC (Ramsey et al. 2000) CR cohorts.

To assess the impact of CR on immune senescence in rhesus monkeys, it was first necessary to characterize the aging of the immune system in this species. Several studies have shown that with increasing age, rhesus monkeys experience a progressive loss of naïve T cells (Pitcher et al. 2002; Jankovic et al. 2003; Messaoudi et al. 2006a; Cicin-Sain et al. 2007). In these studies naïve and memory T cells were defined based on the expression of two surface markers: CD28 and CD95. Using these markers, naïve cells were defined as $\mathrm{CD95^{neg}CD28^{pos}}$, central memory (CM) cells were defined as $\mathrm{CD95^{pos}CD28^{pos}}$ and effector memory (EM) cells were defined as $\mathrm{CD95^{pos}CD28^{neg}}$. As described for humans, advanced age resulted in the loss of naïve T cells and concomitant accumulation of memory phenotype T cells, especially EM ($\mathrm{CD28^{neg}}$) CD8 T cells in rhesus monkeys. Several factors contribute to the loss of naïve T cells in the aged rhesus monkey. Although thymic involution and decreased production of hematopoietic stem cells in the bone marrow undoubtedly are major contributors to the decline in the production of naïve T cells, recent studies suggest that increased T cell turnover in the aged host significantly contributes to the depletion of naïve T cell reserves by increasing their conversion to memory cells (Cicin-Sain et al. 2007). These studies utilized in vivo bromodeoxyuridine (BrdU) pulse-chase experiments. BrdU is a purine analogue that is incorporated into newly synthesized DNA during cell division. Thus, the rate of BrdU uptake and decay can be used to measure the kinetics of T cell proliferation. BrdU incorporation as well as the expression of the cell cycle marker Ki-67 were elevated in peripheral aged naïve T cells compared to adult naïve T cells, indicative of increased homeostatic proliferation (Cicin-Sain et al. 2007). In fact, the rate of proliferation was inversely related to the frequency of naïve T cell. These data are consistent with clinical studies that reported an increase in naïve CD4 T cell proliferation with age (Naylor et al. 2005). Furthermore, as described for humans (Schwab et al. 1997), advanced age results in the appearance of T cell clonal expansions (TCE) in rhesus monkeys (Messaoudi et al. 2006a). Interestingly, the frequency of naïve T cells was inversely correlated to the frequency of TCE in aged monkeys (Cicin-Sain et al. 2007).

Given that increased production of inflammatory cytokines is another hallmark of immune senescence (Zanni et al. 2003), the changes in frequency of memory T cells that secrete inflammatory cytokines IFNγ and TNFα was investigated. In these experiments peripheral blood mononuclear cells (PBMC) were stimulated with anti-CD3 in the presence of Brefeldin A, a chemical that blocks secretion. The cells were then stained with surface antibodies directed against CD4, CD8, CD28 and CD95 to delineate naïve and memory T cell subsets as described in Fig. 4.1.

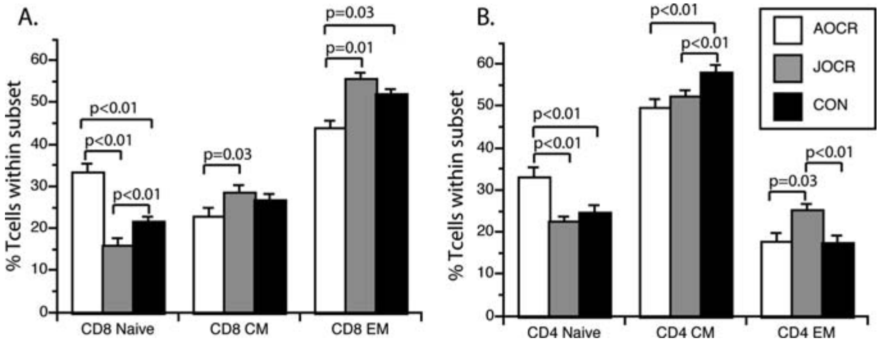

Fig. 4.1 Only CR initiated during early adulthood inhibits decrease in naïve T cell frequency. Percentages of naïve, CM and EM subsets in CD4 and CD8 T cells found in peripheral blood in juvenile-onset CR (JOCR), adult-onset CR (AOCR) and age-matched control male rhesus macaques (CON). Whereas AOCR resulted in an increase in frequency of naïve CD4 and CD8 T cells, JOCR results in accelerated loss of naïve CD8 T cells, and the accumulation of EM CD4 and CD8 T cells. For all panels, mean values are shown for each group at four time points over a 42-month span. Statistical analysis was carried out using the mixed-effects model and significance is denoted by two-sided *P* values above the *bars* (Reprinted from Messaoudi et al. 2008)

Finally, the cells were fixed and permeabilized, and produced cytokines that were visualized by the addition of antibodies directed against IFNγ and TNFα. Using this approach, it was shown that, as described for humans, the number of T cells that secrete these inflammatory cytokines increased with age (Jankovic et al. 2003). Loss of T cell proliferative capacity is another hallmark of immune senescence; thus, age-related changes in T cell proliferation were investigated. In these experiments PBMC were labeled with carboxy-fluorescein diacetate-succinimidyl ester (CFSE) and stimulated with anti-CD3 (Hasbold et al. 1999). CFSE diffuses freely into cells and is cleaved into a fluorescent, membrane impermeable dye whose intensity decays by 50% with every cell division. This flow cytometry based technique allows measurement of the frequency of proliferating CD4 and CD8 T cells subsets simultaneously, the number of divisions, and the percentage of T cells within a given division (Jankovic et al. 2003). The analysis revealed that aged animals show reduced T cell proliferative capacity. In summary, as they age, the T cell compartment in rhesus monkeys undergoes several changes associated with immune senescence in a manner highly analogous to that described in humans.

Several studies have investigated the impact of CR on immune function in both the NIA (Nikolich-Zugich and Messaoudi 2005; Messaoudi et al. 2006b, 2008) and WNPRC CR cohorts (Roecker et al. 1996; Weindruch et al. 1997). CR studies in rodents reported lymphopenia both in peripheral blood as well as lymphoid organs such as the spleen. Thus, early studies in rhesus monkeys investigated whether CR in this species would also result in lymphopenia. Data from these early studies were contradictory, reporting that CR animals exhibit lymphopenia in some studies (Grossmann et al. 1995; Weindruch et al. 1997), but no change in lymphocyte number in another study (Roecker et al. 1996). However, when some of these very same animals were analyzed after >14 years of CR, there was no indication of lymphopenia. The whole white blood cell counts (WBC), as well as lymphocyte numbers, were similar in the CON and CR animals (Messaoudi et al. 2006a, 2008). Upon careful examination of the earlier data, it became obvious that significant differences in lymphocyte numbers were detected at only one time-point and that by year seven post CR, lymphocyte numbers were comparable between CON and CR animals (Weindruch et al. 1997). Thus, it is possible that lymphopenia is a transient effect of CR. A follow-up study examined whether CR modulated the severity of T cell senescence in male and female rhesus monkeys that have been on CR since early age (1–2 years), early adulthood (5–7 years) or advanced age (>17 years) (Nikolich-Zugich and Messaoudi 2005; Messaoudi et al. 2006a, 2008). At the time of analysis, all animals had been on CR for approximately 14 years. The severity of immune senescence was assessed based on parameters defined within the IRP: frequency of naïve T cells; frequency of inflammatory cytokine-secreting T cells; and proliferative capacity of T cells.

Analysis of PBMC from CR males and females (CR initiated during early adulthood) revealed that these monkeys exhibit elevated percentages of naïve CD4 and CD8 T cells compared to CONs (Fig. 4.1). More importantly, the increase in naïve

T cells was paralleled by an equivalent decrease in the CD28^{neg} EM CD8 T cell subset. Given that the accumulation of this subset is a hallmark of immune senescence, these findings suggest that CR can delay the aging of the T cell compartment in rhesus monkeys. To confirm that CR resulted in increased frequency of naïve T cells, the frequency of T cell receptor excision circles (TRECs) was measured. These episomal DNA circles are the result of gene recombination events at the T cell receptor (TCR) chain loci. TRECs are useful markers for identifying and quantifying naïve T cells because they are exclusively of thymic origin and they are stable over time since they do not replicate when naïve T cells divide. This analysis revealed that T cells isolated from CR monkeys contained a higher level of TRECs than T cells from CONs. An increase in naïve T cells should result in enhanced T cell repertoire diversity. To test this possibility, the size diversity of the complementarity-determining region 3 (CDR3) region on the TCR β chain was analyzed. The CDR3 sequence is the most variable portion of the TCR since it the result of gene recombination events. Indeed, the T cells from CR monkeys exhibited a highly diverse CDR3 length profile, whereas, T cells from CON monkeys often showed skewed distribution and restricted CDR3 length profile. Taken together, these data strongly suggest that CR resulted in the delay of naïve T cell loss. Increased prevalence of naïve T cells and reduced frequency of EM T cells has been associated with increased lifespan in mice (Harper et al. 2004) and humans (Larbi et al. 2008).

The increase in naïve T cells should also theoretically result in enhanced T cell proliferative potential. To test this hypothesis, T cell proliferation in response to CD3 stimulation by CFSE dilution was measured (Messaoudi et al. 2006a). Results showed that CR initiated during early adulthood does indeed maintain the ability of T cells to proliferate in response to CD3 polyclonal stimulation. The percentage of cells that remained undivided was consistently found to be lower in CR animals than CON. This difference was also true for the CD28^{neg} EM subset that may contain senescent cells. Taken together with the T cell subset analysis data, these findings indicate that CR not only reduces the percentage of CD28^{neg} T cells, but also improves the proliferative capacity of this subset. The exact mechanisms by which CR preserves naïve T cells are not fully understood. Naïve T cells could be maintained by changes in peripheral factors, such as interleukin 7, a cytokine that plays a critical role in naïve T cell survival through the upregulation of anti-apoptotic molecules. On the other hand, CR could preserve the ability of stem cell progenitors to migrate to the thymus or thymic output into advanced age. Alternatively, a combination of these mechanisms could contribute to the increased frequency of naïve T cells in CR rhesus monkeys.

Given that increased systemic inflammation is a hallmark of immune senescence, several studies investigated CR-induced changes in inflammatory cytokine levels in aged rhesus monkeys. IL-6 was extensively studied since plasma levels of IL-6 are significantly elevated in aged individuals (Rink et al. 1998; Wikby et al. 2006) and have been associated with increased morbidity and mortality in the elderly (Ershler and Keller 2000; Bruunsgaard 2002; Huang et al. 2005). CR was shown to reduce the steady-state plasma levels of IL-6 in rhesus monkeys (Lane et al. 1995), as well as IL-6 production by PBMC following LPS stimulation or induction of oxidative damage (Kim et al. 1997). In contrast to these results, a later study found that CR did not impact IL-6 secretion by PBMC following LPS stimulation in male rhesus monkeys (Mascarucci et al. 2002). The discrepancies between these two studies could be due to differences in the age of the animals studied: the animals in the early study were older adults (9–15 years of age); whereas, the latter study looked at young (4–5 years) and old (17–23 years) separately. The duration of CR also differed between the two studies (5 years vs. 2 years). Nevertheless, these data suggest that steady levels of IL-6 are reduced in CR animals, but the ability of their immune cells to secrete IL-6 once challenged is not altered. This distinction is critical since production of IL-6 can play an important role in the generation of the B cell response to bacterial infections (Hilbert et al. 1989) and the body's response to trauma (Akira et al. 1993). These studies suggest that CR reduces systemic inflammation, but does not interfere with the body's ability to respond to bacterial challenge. CR also reduced IL-1β and IL-8 production by PBMC in response to oxidative stress (Kim et al. 1997). In addition, it was investigated whether CR reduced IFNγ and TNFα production by T cells in response to anti-CD3 stimulation, which was found to be elevated in aged macaques (Jankovic et al. 2003). Results indicated that adult onset CR resulted in a decreased frequency of CM and EM T cells that secreted IFNγ and TNFα in response to CD3 stimulation (Messaoudi et al. 2006a) (Fig. 4.2).

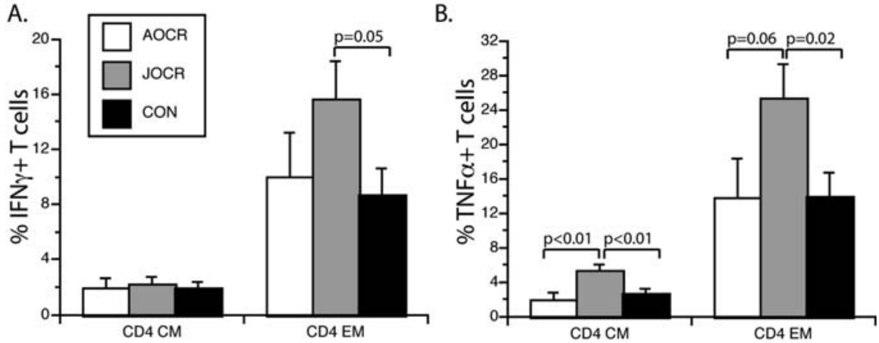

Fig. 4.2 CR initiated during adulthood decreases frequency of IFNγ and TNFα producing memory CD4 T cells. Percentage of CM and EM CD4 T cells that secrete IFNγ (**a**) or TNFα (**b**) in response to a 6 h in vitro stimulation with anti-CD3 was determined by intracellular cytokine staining. In contrast to AOCR, JOCR results in increased prevalence of inflammatory cytokine-secreting memory T cells (Reprinted from Messaoudi et al. 2008)

These findings differed from earlier studies that showed that IFNγ production in response to phytohematogluttinin (PHA) stimulation was higher in PBMC isolated from young and aged CR animals compared to age-matched controls (Mascarucci et al. 2002). However, those studies were carried out after 2 years of CR and did not specifically measure IFNγ production by T cells, but rather by total PBMC. Thus, it is possible that macrophages from CR animals have increased IFNγ production, or that short-term CR induces an increase in IFNγ secretion by PBMC. These data suggest that as described in mice, CR can reduce the production of inflammatory cytokines by different types of immune cells. These findings strongly suggest that adult-onset CR delays immune senescence and should preserve immune function in aged animals.

There were significant discrepancies between earlier and more recent CR studies in rhesus monkeys with regards to T cell proliferation and cytokine production. The duration of CR as well as the age at onset differed significantly between the earlier studies and more recently reported data. The latter studies examined monkeys that started CR during early adulthood and were maintained continuously on this regimen for 14 years; whereas, earlier studies examined monkeys after a short period of CR (~2–7 years) and whose age at onset was about 8–14 years. To examine how these variables (age at onset, length of CR) modulate the impact of CR on T cell senescence, several immune senescence parameters were measured in monkeys initiated to CR either early (1–2 years) or late (>17 years) in life (Messaoudi et al. 2008).

The analysis revealed that in sharp contrast to the results seen with adult-onset CR (AO-CR), juvenile-onset CR (JO-CR) in male rhesus monkeys resulted in accelerated loss of naïve T cells and the accumulation of terminally differentiated EM CD4 and CD8 T cells (Messaoudi et al. 2008). Consequently, T cell repertoire diversity was reduced in JO-CR male rhesus monkeys with an increase in frequency in T cell clonal expansions, which was previously shown to be a predisposing factor for reduced immune response to infection (Messaoudi et al. 2004). Furthermore JO-CR increased the frequency of CD4 and CD8 memory T cells that secreted IFNγ and TNFα in response to CD3 stimulation. Moreover, JO-CR resulted in reduced T cell proliferative capacity. A higher frequency of T cells from JO-CR male rhesus monkeys remained undivided compared to age-matched CONs and AO-CR animals. To assess the extent of this loss in proliferative capacity, a geometric division score was assigned that is derived from the percentage of the cells in each division and gives more weight to cells that have undergone multiple rounds of division. T cells from AO-CR animals have a positive geometric score indicative of a significant percentage of cells that underwent several (>3) rounds of proliferation. T cells from JO-CR animals, on the other hand, had negative scores since most of the dividing cells remained in the first division. These observations were similar to earlier studies that reported that JO-CR resulted in reduced T cell proliferation to pokeweed mitogen, concanavalin A, and PHA (Weindruch et al. 1997), and diminished calcium response to CD3 stimulation (Grossmann et al. 1995). Overall, these data indicate that the initiation of CR in juvenile male rhesus monkeys exacerbated, rather than delayed, T cell senescence.

Interestingly, in female rhesus monkeys, JO-CR resulted in decreased prevalence of EM CD8 and CD4 T cells and a significant increase in naïve CD8 T cells. Although decreased frequency of EM T cells would in theory indicate delayed immune senescence, female monkeys subjected to JO-CR displayed increased frequency of IFNγ and TNFα secreting T cells and showed no benefit from CR when proliferative responses were measured. These results highlight the importance of measuring multiple phenotypic and functional parameters when evaluating and interpreting the impact of an intervention on immune senescence. Despite the observed difference between male and female JO-CR animals, the data strongly suggest that JO-CR at best has no impact on immune senescence and at worst can accelerate the aging of the T cell compartment. The difference in JO-CR impact on memory T cell frequency between male and female monkeys could be a result of gender differences in aging or the fact that the female cohort was, on average, 5 years younger than the males. Therefore, loss of naïve T cells could be more pronounced between 15 and 20 years of age and it is possible that in 5 years the JO-CR females would experience a more rapid loss of naive T cells than age-matched CONs. It is worth noting that JO-CR resulted in a more significant delay in skeletal muscle development and weight gain in males than females (Lane et al. 1999). This delay in development was accompanied with a more pronounced reduction in abdominal trunk fat, with males experiencing a three-fold decrease and females experiencing only a two-fold decrease compared to CONs (Lane et al. 1999). Such gender-specific differences in response to CR could impact lymphopoeisis, lymphocyte homeostasis and function as well. To determine whether these differences are due to the lower age of the females, the PBMCs of the JO-CR females will have to be reanalyzed when they reach the same age as the males were when they were analyzed.

The recent immune studies also indicated that old onset CR (OO-CR) resulted in reduced T cell proliferative response to stimulation in the absence of any detectable changes in T cell subset distribution. Other measurements in this cohort also revealed trends towards worsening of immune risk phenotype. Given that nutritional stress of CR is likely to have other unwanted effects upon the physiology of old primates (Ingram and Mattison, unpublished observations), extreme caution should be exercised when initiating late-life CR. Overall, these results suggest that there is an optimal window for the initiation of CR. The delay of immune senescence by AO-CR would theoretically lead to improvement; whereas, JO-CR would be expected to result in a diminished immune response to infection or vaccination. Although earlier reports suggested a decrease in immune response to vaccination in CR rhesus monkeys, studies are currently specifically investigating the impact of AO-CR versus JO-CR on the immune response to vaccination (Roecker et al. 1996; Weindruch et al. 1997).

Since Walford et al. (1973) first reported the beneficial effects of CR on immune function in 1973, a great number of investigators have demonstrated in mice and more recently in rhesus monkeys that CR can delay immune senescence. However, CR immuno-enhancing effects in rhesus monkeys are modulated by the age at which CR is initiated as well as the duration. Adult onset of CR shows a uniquely beneficial impact upon the aging of the T-cell compartment when maintained for the life of the monkey (Messaoudi et al. 2006a). In contrast juvenile or late onset CR can accelerate T cell senescence (Messaoudi et al. 2008). Additional studies will be required to evaluate the relative importance of these benefits against the increase in lifespan and improved function of other organs or systems.

4.5 CR and Behavior

Although CR has been demonstrated to increase longevity in a number of animal species, less is known about the effects of CR on behavioral indices that decline with age. In recent years, both the NIA and WNPRC have increased efforts to develop age-sensitive testing strategies to gain a better understanding of the effects of CR on behavioral function in rhesus monkeys. The NIA and WNPRC employ similar devices and testing procedures to study behaviors that have been reported to be age-sensitive in NHPs, such as short-term memory, home-cage activity, and fine-motor performance and how these can be affected by CR.

4.5.1 Motivation

It is frequently asked whether or not CR animals, regardless of the length of restriction, are more satiated than their CON counterparts. As a first step in addressing this concern, NIA examined food motivation in 65 monkeys from the long-term CR project which had been on their respective diet for at least 5 years. The results of this study would ultimately lay the foundation for future behavior projects. This particular task was important for future behavioral projects because it determined if the monkeys, without any additional food restriction, would perform a behavior task for a small food reward and it provided evidence of the CR monkeys' satiation, and thus their motivation to work for a small food reward in comparison to the CON monkeys.

Motivation was defined as the time spent trying to retrieve a food item that was unattainable. The monkeys were trained to retrieve a food biscuit from a specially designed apparatus attached to their home cage. Once trained, motivation was tested by placing the reward behind what was now a locked clear panel making it irretrievable. The time that the monkey spent pushing against the clear panel to retrieve the biscuit was recorded as a measure of motivation (Fig. 4.3) (Mattison et al. 2005).

This particular task, requiring the monkey to initiate and maintain the behavior necessary to retrieve the food reward, is a function of activational motivation (Salamone and Correa 2002). Although there was a significant age-related decline in motivation for food, the results from this motivation task indicated that the CR monkeys did not spend more time attempting to retrieve the food item compared to CON monkeys (Mattison et al. 2005). Thus, based on these results, it would appear that CR monkeys were not more motivated for food compared to CON. This lack of a diet-effect for food motivation was crucial in devising a behavior regimen that could make comparisons between age groups, as well as diet groups.

4.5.2 Cognition

Normal human aging is associated with declines in the ability to remember (Levine et al. 2002), to acquire new information (Small et al. 1999), and executive function

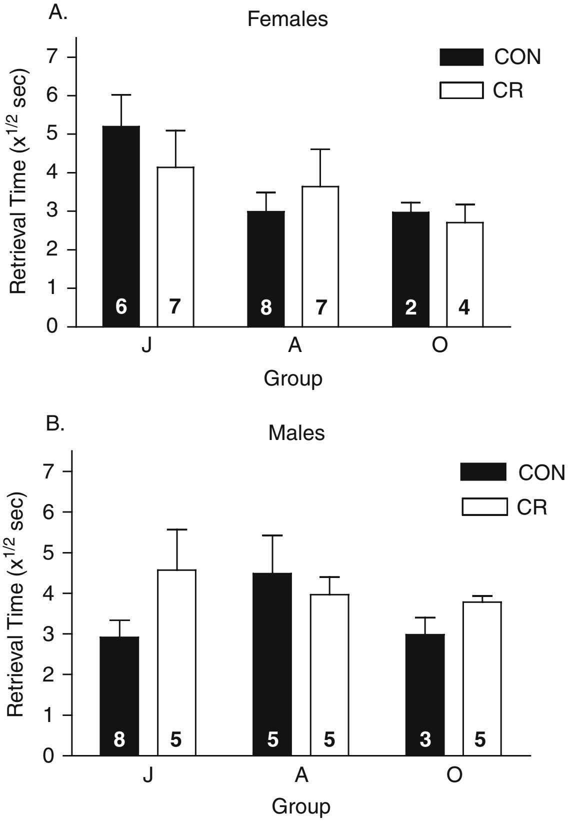

Fig. 4.3 CON and CR monkeys were equally motivated on a food reward task. Retrieval time in a food motivation task (mean±SEM) for (**a**) female and (**b**) male rhesus monkeys in different age groups on a CON or CR diet for over 8 years. The number of monkeys tested is indicated in the respective *bar* (Reprinted from Mattison et al. 2005)

(Albert 1993). Similarly, monkeys experience an age-related decline in several domains of cognitive function, including short-term memory (Rapp and Amaral 1989; Kubo et al. 2001; Buccafusco et al. 2002) and executive system function (Bartus et al. 1979; Rapp 1990). Because rhesus monkeys do not develop certain neuropathological hallmarks of Alzheimer's disease (Peters et al. 1996), they are an excellent model for studying the neurobiology underlying the behavioral effects of aging.

An important question to be addressed in both the NIA and WNPRC studies is the effect of CR on brain and cognitive function. In rodent studies, findings generally support the view that CR attenuates age-related decline in performance in learning and memory tasks (Ingram 1991). At NIA, a simple learning task, two-choice discrimination, was used to study the effect of long-term CR on learning and memory in the monkeys. Cognitive flexibility was assessed by evaluating the monkeys' performance on the reversal of the two-choice object discrimination. Previous reports of object discrimination tasks have found either a significant age-related decline (Voytko 1999) or no effect of age (Bartus et al. 1979; Rapp 1990) while object or position reversal learning consistently declines with age (Bartus et al. 1979; Rapp 1990; Tsuchida et al. 2002).

Monkeys were trained on both two-choice object discrimination and object reversal tasks with a modified Wisconsin General Testing Apparatus (WGTA) that attached to a monkey's home cage. For the object discrimination task, the monkey learned to recognize a familiar object for a reward. In the reversal task, the reward contingencies were the opposite and the previously unrewarded object became the rewarded object. For both tasks there was a significant positive correlation between age and number of errors among the CON monkeys. These results indicate an age-related increase in response rigidity. However, there were no significant differences between diet groups for young or old monkeys in either task (unpublished observations) (Fig. 4.4).

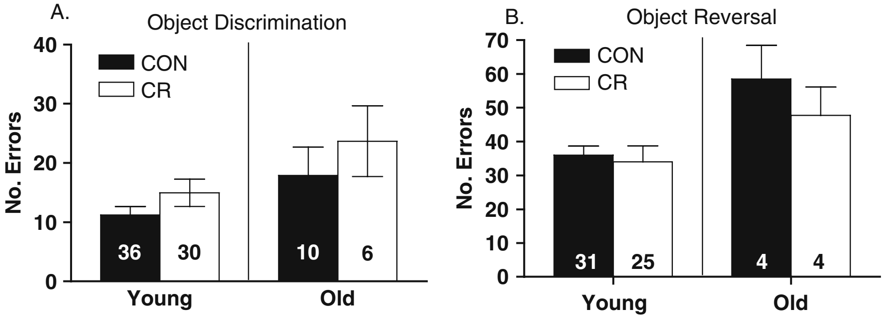

Fig. 4.4 Performance on an object discrimination and reversal task was not affected by CR. The number of errors made in a task of object discrimination (**a**) and object reversal (**b**) for young (16.1+0.34 years) and old (29.8+0.59 years) monkeys on a CON or CR diet. The number of monkeys tested is indicated in the respective *bar* and includes both males and females. There were no significant differences between diet groups

The lack of a diet group effect on the Object Discrimination task reinforces that CON and CR monkeys have the equivalent ability and motivation to meet the performance requirements, i.e., use their vision to discriminate between 3-dimensional objects in order to obtain a food reward.

More complex cognitive tests at the NIA and WNPRC are currently underway with a custom-designed Visual Basic computer program, the Yerkes Cognition Battery (YCB) (Designed by Dr. Jim Herndon, Yerkes Primate Center) that requires the monkeys to make responses on a touch screen.

4.5.3 Activity

A decline in the level of general locomotor activity is one of the most sensitive and robust age-related behavioral changes (Ingram 2000; Sallis 2000) and has been recorded in numerous animal species, including nonhuman primates (Emborg et al. 1998; Zhang et al. 2000) and humans (Ingram 2000). Several different automated devices have been used to measure activity in nonhuman primates, including ultrasonic motion sensors (Kemnitz et al. 1993; Weed et al. 1997; Moscrip et al. 2000) videotape recordings (Walton et al. 2006), biotelemetry (Line et al. 1989), and accelerometers (Papailiou et al. 2008). By using automated devices, locomotor activity can be measured in a highly standardized, noninvasive manner.

NIA had two early reports of locomotor activity. Following 6 years on the study, an age-related decline in activity for both CON and CR males was evident, but an increase in activity with CR was evident in only those monkeys in the mid-age range group (Weed et al. 1997). Similarly, Moscrip et al. (2000) reported an age-associated decline in activity in a cross-sectional analysis of the females. However, in this case, among the youngest group of monkeys (6–8 years), the CR females were less active than age-matched CONs. From WNPRC, at both 6 and 12 months on study, activity in the CON males had increased from baseline while it decreased in the CR group (Kemnitz et al. 1993), but by 30 months there were no differences in activity between the diet groups (Ramsey et al. 1997).

Both NIA and WNPRC studies have adopted the use of an actigraphy-based data system with the Actical monitor and Actiwatch, respectively (Respironics, Inc., Bend, OR) for present and future studies of motor activity. The devices are compact, lightweight and worn on standard nylon or aluminum primate collars (Primate Products, Inc., Woodside, CA). The most recent data from NIA evaluating activity after 14–17 years on study confirms an age-related decline regardless of diet group. The young CR monkeys appear to be more active than their age-matched CONs; however, this is not apparent in the old group (unpublished observation). The longitudinal analysis of this parameter continues, but it is clear the findings are complicated by differences in diets, age, gender, animal history, length of CR, health, genetics, and other factors.

4.5.4 Fine-Motor Performance

Similar to humans, rhesus monkeys possess dexterous hands capable of precision grips (Roy et al. 2000). Aging effects on motor function in NHPs have been contradictory. Some earlier studies found no effect of age (Bachevalier et al. 1991; Arnsten et al. 1995) while the more recent reports suggest a slowing of motor function (Emborg et al. 1998; Grondin et al. 2000; Zhang et al. 2000; Lacreuse and Herndon 2003). Currently, very little is known about the consequences of CR on motor behavior involving prehension.

Procedures are currently underway at both the NIA and WNPRC to examine the longitudinal effects of CR on motor performance in aging colonies of rhesus monkeys. Both sites are using a motor assessment panel originally designed to examine coarse- and fine-motor performance in humans with Parkinson’s disease but modified for use with rhesus monkeys (Gash et al. 1999). This apparatus and similar versions have been used to reproducibly and reliably measure age-related declines in humans (Gash et al. 1999; Smith et al. 1999, 2005) and rhesus monkeys (Gash et al. 1999; Grondin et al. 2000; Zhang et al. 2000; Lacreuse and Herndon 2003; Lacreuse et al. 2005; Lacreuse 2006; Cass et al. 2007). This monkey Movement Assessment Panel, or mMAP apparatus, evaluates movement of the forearm during reaction and withdrawal (coarse motor) and movement of the hand during object manipulation (fine motor). Monkeys were trained and tested on three motor tasks of increasing difficulty: level platform, straight rod, and hook.

Data from the NIA monkeys confirms an age-related decline in motor function in both CON and CR groups. As expected, performance times for fine-motor tasks increased with increased task difficulty. Additionally, CR monkeys tended to be faster than age-matched CONs (unpublished observations). Longitudinal testing will determine if this superior dexterity in the CR group is maintained and possibly related to osteoarthritis.

4.6 Short-Term CR and Neuroprotection

The impact of a short-term CR regimen was studied in a group of rhesus monkeys injected with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induces a Parkinson's-like syndrome by selective degeneration of dopamine (DA) neurons and resultant neurochemical and histopathological alterations with motor dysfunction. Six adult male rhesus monkeys were maintained at approximate *ad libitum* levels of food intake while seven age-matched monkeys were on CR to approximately 70% of their *ad libitum* intake. After 6 months on the diet regimen, the monkeys were unilaterally injected with MPTP; mMAP and locomotor activity were assessed after ample recovery time. Monkeys were maintained on their respective diet throughout the study and euthanized approximately 4 months after the MPTP injections (Maswood et al. 2004).

MPTP treatment produced a nearly 12-fold unilateral depletion of DA neurons in the striatum. Measures of mean movement distance and speed of movement were significantly reduced in both diet groups following the injection; however, the CR monkeys were more than 2-fold more active than the CON group with generalized activity, not stereotypic circling behavior. Prior to experimental interventions, all monkeys were trained to be proficient on the mMAP using both limbs for all three tasks. Although lacking statistical significance, the CR monkeys performed better following MPTP and exhibited less severe motor impairment than the CON group. In fact, only one of six control monkeys could perform the hook task while four of seven CR monkeys were successful. CR monkeys performed better on the most difficult hook task than the CON monkeys did on the simplest platform task.

Additionally, the neurochemical data indicated a protective effect of the CR diet. Monkeys on CR had higher levels of dopamine and dopamine metabolites in affected brain regions compared to CON. Glial derived neurotrophic factor and to a lesser extent brain-derived neurotrophic factor were higher in the striatums of the CR monkeys. These findings suggest that a dietary manipulation such as CR, can attenuate neurotoxic effects and warrant further investigation as a neuroprotective mechanism.

4.7 Morbidity and Mortality

Very recently, the WNPRC study has reported a lower incidence of age-related deaths in CR monkeys after more than 20 years of study (Colman et al. 2009). Furthermore, the onset of age-associated diseases was significantly reduced in the CR monkeys as conditions such as cancer, cardiovascular disease, glucoregulatory impairment, diverticulosis, and arthritis were detected at one-third the rate as in control monkeys. It may be many more years before it is known if CR might increase maximal lifespan in a primate species but the findings are consistent with what has been demonstrated in many other species and suggest that CR could reduce the incidence of age-related morbidities in humans.

4.8 Conclusion

Because of their similarity to humans, monkeys provide the best available opportunity to investigate aging processes as well as the efficacy of interventions aimed at improving quality of life for older individuals. CR is the only intervention shown to consistently increase median and maximal lifespan. However, the impact of CR on long-lived primates such as humans remained poorly understood until recently. Our understanding of aging processes and the effect of CR has increased dramatically over the last decade as a product of the studies still underway at the NIA and WNPRC. These data indicate that rhesus monkeys exhibit many beneficial effects of CR and, although not yet conclusive, benefits for both health and lifespan are emerging. It is also becoming clear that the effect of CR is affected by both the age of CR onset and duration. The mechanisms by which CR exerts its effect remain unknown, and further studies are needed to elucidate these mechanisms.

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Chapter 5
Dietary Restriction and Aging in *Drosophila Melanogaster*

Luc Poirier, Rafael de Cabo, and Sige Zou

5.1 Introduction

Dietary restriction (DR) or calorie restriction (CR), the reduction of nutrient intake without causing malnutrition, results in the extension of life span in a diverse array of organisms and may therefore be regulated by evolutionary conserved mechanisms. *Drosophila melanogaster* is a simple model organism that is relatively easy to work with and inexpensive to maintain. It has been thoroughly characterized at the genetic and genomic level and has a relatively short life span (Helfand and Rogina 2003; Poirier and Seroude 2005). Furthermore, the simple sugar/yeast (SY) food that *Drosophila* is routinely reared on allows for the easy manipulation of the food components in DR experiments. These features have made *Drosophila* a widely used model organism in the study of the mechanisms of DR and aging. Information gathered through experiments with *Drosophila* may help with the identification of molecular targets for therapeutic interventions to slow the aging process in higher organisms including humans.

5.2 DR Paradigms in *Drosophila Melanogaster*

The efficacy of extending *Drosophila* life span via DR has been firmly established (Partridge et al. 2005; Piper and Bartke 2008). Two methods of eliciting DR responses in *Drosophila* are commonly used (Partridge et al. 2005; Bass et al. 2007a). The first method consists of rearing flies on food where all of the nutrients have been diluted, thereby decreasing the concentration of all the components in the food (Fig. 5.1).

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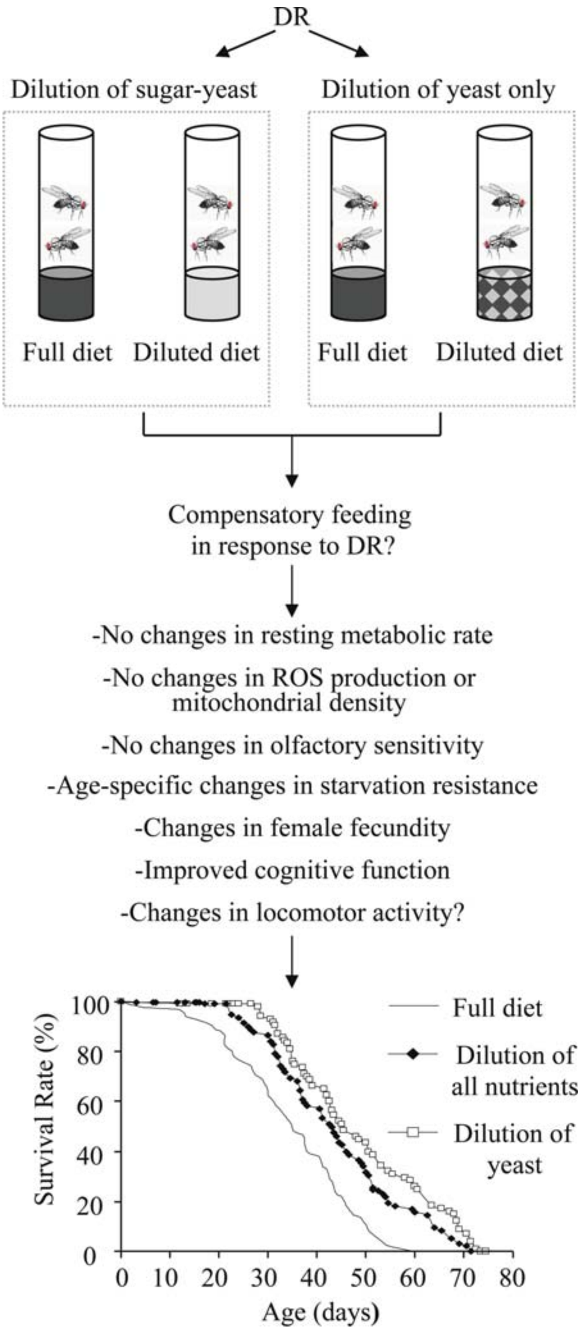
DR
Dilution of sugar-yeast
Dilution of yeast only
Full diet
Diluted diet
Full diet
Diluted diet
Compensatory feeding in response to DR?
-No changes in resting metabolic rate
-No changes in ROS production or mitochondrial density
-No changes in olfactory sensitivity
-Age-specific changes in starvation resistance
-Changes in female fecundity
-Improved cognitive function
-Changes in locomotor activity?
Survival Rate (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Age (days)
Full diet
Dilution of all nutrients
Dilution of yeast

Fig. 5.1 Overview of strategies used to implement DR in *Drosophila melanogaster* and the changes in behaviors, functions and life span that results from DR

Within a certain range of concentrations, the dilution of all nutrients (e.g., from 1.5x to 0.5x of a base SY diet with 10% sugar and 10% yeast extract) has been shown to extend *Drosophila* life span (Chapman and Partridge 1996). This prolongevity effect, however, has been shown to depend on the strain of fly and sources of ingredients, especially yeast extract, in the food. In addition, it is difficult to rule out that the prolongevity effect of DR is due to a reduction of a toxin in the food or a food concentration-related toxic effect. To address these issues, attempts have been made to standardize the DR diets to meet the following criteria. Based on research done in other model organisms, food regimens in *Drosophila* that elicit true DR effects should: (1) result in an increase in life span with a concomitant decrease in fecundity when nutrient levels are reduced, and, (2) result in a decrease in life span with a concomitant increase in fecundity when nutrient levels are augmented (Klass 1977; Holehan and Merry 1985; Chippindale et al. 1993; Chapman and Partridge 1996). Manipulations of food concentrations that modulate life span but not fecundity in the expected manner are not true DR effects and presumably arise from other factors such as food-related toxins or nutritional imbalance. Using these criteria, it has been proposed that a standard DR protocol should: (1) use whole cell lysates prepared from Brewer's yeast, (2) contain sucrose at a concentration of at least 50 g/L, and, (3) optimize the range of nutrient dilution for each particular fly strain (Bass et al. 2007a). It should be noted that since reproduction and lifespan are not tightly linked, the currently proposed DR standard may overlook DR effects that are unrelated to reproduction.

Although the dilution DR approach robustly extends longevity, it is uninformative with regards to which nutrients, if any, are the essential modulators of life span. The second DR method has been employed to address this issue by manipulating individual nutrients while the concentrations of the remaining food ingredients are kept constant or the total calorie content in the food are kept constant (Fig. 5.1). Studies from the early 1990's of flies fed different amounts of yeast paste (1.5 and 0.15 mg of yeast paste daily as high and low yeast foods to a population of flies, respectively) have shown that a reduction in yeast intake elicits an extension of life span in *Drosophila* (Chippindale et al. 1993). In recent experiments, isocaloric food media containing different concentrations of yeast and sugar have confirmed that the reduction in yeast content, not in calorie content, is the primary determinant of life span in response to DR. However, this conclusion is based on findings that flies do not modulate their feeding behavior in response to dietary conditions, but these findings have been challenged on the basis of other experimental data (Carvalho et al. 2005; Mair et al. 2005; Min and Tatar 2006a; Min et al. 2007).

Since yeast is the sole protein source for flies reared on standard SY food, experiments have been conducted to see if a reduction in the level of amino acids is sufficient to elicit an extension of life span in *Drosophila*. Indeed, restricting amino acid content does increase life span, demonstrating that the level of amino acids is sufficient to regulate *Drosophila* longevity (Min and Tatar 2006b). However, although restriction of individual amino acids (e.g., trytophan, methionine) can lead to an extension in life span in rodents, restriction of methionine alone has no effect on *Drosophila* lifespan (Troen et al. 2007). It remains to be determined whether restriction of other individual amino acids can lead to lifespan extension in *Drosophila* (De Marte and Enesco 1986; Orentreich et al. 1993; Richie et al. 1994; Zimmerman et al. 2003).

Two recent nutritional geometry studies have demonstrated that nutrient-dependent life span determination in *Drosophila* is not mediated solely by caloric intake, but rather is primarily determined by the relative amounts of protein and carbohydrate consumed (Lee et al. 2008; Skorupa et al. 2008). Lee et al. (2008), for example, found that diets with low protein to carbohydrate (PC) ratios elicit longer life spans in *Drosophila*. Even though a strict trade-off between longevity and reproduction is not evident, these two fitness measures are maximized on diets with different PC ratios, with longevity and reproduction maximized at 1:16 and 1:4 PC ratios, respectively (Lee et al. 2008). Subsequently, Skorupa et al. reported that a simultaneous increase of both sugar and yeast causes a less severe reduction in life span than when sugar or yeast are increased alone (Skorupa et al. 2008). They have also found that although a high level of yeast in the diet is good for female fecundity, excess yeast negatively affects *Drosophila*'s life span. Furthermore, the detrimental affect of high yeast levels is more prominent at high sugar concentrations. These findings suggest that a balanced protein: carbohydrate diet is the most important nutritional influence on life span.

5.3 Food Intake Under DR

In larger organisms (e.g., Mexican fruitfly, mice, rats and non-human primates), DR can be imposed by simply limiting the amount of food provided. Due to small body size, DR protocols in *Drosophila* involve dilutions of the food medium and consequently can not control for actual nutrient consumption, which therefore has to be measured (Masoro 1988a; 1988b; Carey et al. 2008). Several methods have been established for this purpose. One strategy is to estimate food intake indirectly through parameters that are related to nutrient intake. One such parameter is fecal density, measured as the number of fecal pellets/fly/cm^2/day (Driver et al. 1986). Another indirect approach is to measure the number of times a fly's proboscis makes contact with the food medium in an observed period of time (Mair et al. 2005). Four methods have been designed to directly measure food intake. The first involves the addition of radioisotopes to the food medium, which allows measurement of food intake by quantifying (using a scintillation counter) the level of radioisotope incorporation in flies after a feeding period (Carvalho et al. 2005). The second method involves spiking the food media with stable dyes (e.g., Blue No.1) (Edgecomb et al. 1994). The level of dye intake and hence of food consumption is determined by homogenizing the flies and measuring absorbance with a spectrophotometer. A third method involves measuring the caloric flux (the proportion of caloric intake actually put to physiological use during a given period of time) in flies reared on different diets (Min et al. 2007). The fourth method is known as the Capillary Feeder (CAFE) system and uses capillary tubes for feeding (Ja et al. 2007; Lee et al. 2008). The capillary tubes allow direct measurement of the volume of consumed food (Carvalho et al. 2005).

Each of these strategies has strengths and weaknesses. While measuring the frequency of proboscis/food contact is straightforward, this method assumes that the volume of food consumed at each contact event is equivalent. This assumption has not been experimentally verified. The radioisotope labeling method only measures nutrient assimilation, ignoring food that is ingested but excreted rather than incorporated into biomass. In addition, these measurements may be confounded by different food retention rates between DR and fully-fed flies (Wong et al. 2008). Similar issues surround the use of stable dyes to measure food intake, where typically only the dye in the gut is measured by spectrophotometry. Furthermore, measurements of food intake using dyes is not as sensitive as radioisotope based methods, and questions remain as to whether or not dyes are an appropriate way to measure food consumption. Caloric flux can be an accurate means of quantifying nutrient intake as long as all of the calories consumed are accounted for, including calories burned in metabolism, invested in biomass and excreted as fecal matter (Piper et al. 2007). Of the four methods, only the CAFE system directly measures actual food consumption. However, since the CAFE system relies on liquid media, and because the flies must eat in an upside down position, which is different from standard fly culture conditions, the CAFE method may lead to alterations in feeding rates and/or total food consumption.

In relatively large organisms, such as rodents, the amount of food provided to both fully-fed and DR animals accurately reflects the difference in food intake. Whether or not differences in nutrient concentrations of the food media reflect differences in nutrient intake in *Drosophila* remains unknown. Measurements by fecal density (Driver et al. 1986), radioisotope labeled food intake (Carvalho et al. 2005), dye-stained food intake (Edgecomb et al. 1994; Min and Tatar 2006a; Skorupa et al. 2008) and calorimetric analysis (Min et al. 2007) indicate that DR induces compensatory feeding. However, measurements of the frequency of proboscis contact with food (Mair et al. 2005), and of intake of dye-stained food (Bross et al. 2005), suggest that DR does not induce compensatory feeding. It is clear that more work needs to be done in order to clarify the relationship between food composition and feeding behavior.

5.4 The Effect of DR on Mortality

Mortality trajectory analyses of lifespan data have shed light on the demographic impact of DR. Experiments in which adult flies are switched between control and DR diets indicate that a decrease in nutrient intake affects life span by reducing the acute likelihood of death, without affecting mortality rate (Mair et al. 2005). However, comparison of mortality data from a geometric study with adult flies raised on 28 different diets suggests that DR increases life span by both delaying the onset of mortality and by slowing the increase in the mortality rate thereafter (Lee et al. 2008).

5.5 Physiological and Behavioral Changes Induced by DR

5.5.1 Metabolic Rate and Reactive Oxygen Species

The rate of living hypothesis of aging postulates that an organism's life span is determined by its rate of metabolism, and that within a given species, organisms with higher metabolic rates will have a shortened life span relative to individuals with lower rates of metabolism (Speakman et al. 2002). For example, flies raised at lower ambient temperatures have a lower level of metabolic activity and extended life span, while flies raised at higher temperatures have a higher levels of metabolism and shorter life span (Miquel et al. 1976). To address the effect of DR on metabolism, the body-mass and resting metabolic rate of flies after DR have been measured. DR results in a significant decrease in body mass, but does not change mass-specific metabolic rates (measured using both respirometric and calorimetric assays) (Bross et al. 2005). This is not surprising given that the relationship between metabolic rate and lifespan remains unresolved in *Drosophila* and other species. For example, flies carrying a mutation in the gene encoding the insulin receptor substrate *chico*, which have a reduced body size and are long-lived, do not exhibit a reduction in metabolic activity compared to wild type flies (Hulbert et al. 2004).

Another possibility for how DR extends life span is by decreasing the production of reactive oxygen species (ROS). The oxidative stress theory of aging posits that ROS, produced during oxidative respiration, are a major cause of oxidative damage to cellular macromolecules (Miwa et al. 2003). This macromolecular damage manifests itself as the biological phenomenon of aging. However, measurements of ROS production from mitochondria isolated from thoracic flight muscles do not show a reduction in ROS levels between DR and control flies, although DR does seem to affect mitochondrial morphology (e.g. size) and the activities of some mitochondrial enzymes (Miwa et al. 2004). Alternatively, a reduction in the number of mitochondria in response to DR would lower the level of ROS. Measurement of mitochondrial density using both mitochondrial enzyme based assays and electron microscopy has failed to detect differences in mitochondrial density between DR and fully-fed flies (Magwere et al. 2006). However, research using cybrid fly strains (i.e., strains whose nuclear and mitochondrial genome sources differ) suggests that mitochondria can modulate the response to DR (Soh et al. 2007). Flies carrying mitochondrial types considered to be highly efficient (e.g., "La" type mitochondria) are unresponsive to DR while flies with less efficient mitochondria (e.g., "Ra" type mitochondria) can exhibit extended life span in response to DR (Soh et al. 2007).

5.5.2 Stress Response

Although DR does not seem to reduce the levels of ROS produced by mitochondria (Miwa et al. 2004; Magwere et al. 2006), an increase in the expression of enzymes that protect against these highly reactive molecules might result in a net decrease in oxidative stress. However, no link between DR-dependent life span extension and increased resistance to oxidative stress has been found in *Drosophila*. On the contrary, direct measurement has revealed that DR in older flies significantly decreases resistance to oxidative stress (Burger et al. 2007). Furthermore, both cytosolic superoxide dismutase 1 (SOD1) and mitochondrial SOD2 are unresponsive to DR, contrary to the proposal that DR causes life span extension by increasing resistance to oxidative stress (Kabil et al. 2007).

Despite this apparent lack of DR-mediated increases in the level of protective enzymes and in resistance to oxidative stress, other research shows that DR does delay the accumulation of oxidative damage in *Drosophila*. When the level of lipid peroxides (a product of ROS damage to cellular lipids) is plotted against percent life span, similar peaks in lipid peroxide levels occurs at around 60% of life span in both DR and fully-fed flies. Therefore, although DR and fully-fed flies experience similar amounts of oxidative stress over the life span, this accumulation is delayed in DR flies with respect to chronological time (but not with respect to physiological age), potentially contributing to an extension of life span (Zheng et al. 2005b). Interestingly, this work also suggests that the levels of lipid peroxidation can potentially be used as a biomarker of physiological age (Zheng et al. 2005b).

The level of functional senescence has been measured for various other *Drosophila* traits. DR influences resistance to starvation in an age-specific manner. While young DR flies exhibit an increase in resistance to starvation when compared to controls, DR causes a reduction in resistance to starvation in older flies (Burger et al. 2007). Finally, when compared to fully-fed controls, DR flies show increased resistance to infection to certain bacterial strains as well as a decrease in cold stress resilience (Burger et al. 2007).

5.5.3 Reproduction

Many interventions that extend *Drosophila* life span also decrease female fecundity. Not surprisingly, a number of studies have found that DR reduces age-specific fecundity and lifetime egg production, providing a potential metric for standardizing DR protocols (Chippindale et al.1993; Chapman and Partridge 1996; Bross et al. 2005; Burger et al. 2007). One hypothesis is that DR increases life span by diverting the majority of nutrient resources to somatic maintenance at the cost of reduced reproduction. Using radioisotopes, measurements of nutrient allocation revealed that although fully-fed flies invested more nutrients in somatic maintenance (and reproduction) in gross terms, the ratio of nutrient investment in somatic maintenance relative to reproduction was greater in DR flies (O'Brien et al. 2008). Based on these findings, it might be the case that DR extends life span by a relative increase in investment in somatic maintenance in conjunction with a simultaneous decrease in the level of damage incurred through reproduction, which is greatly reduced by DR. Other aspects of reproduction and mating are also affected by nutrient intake. Several studies have uncovered a diet-dependent cost on female reproduction based on mating condition. On high nutrient concentration diets, females that are continuously exposed to males are less fecund than females that are intermittently exposed to males (Chapman and Partridge 1996; Burger et al. 2007). This cost of mating is not observed on low nutrient concentration diets.

Several studies have shown that DR-mediated extension in life span and corresponding decreases in female fecundity can be uncoupled. Female flies carrying a dominant *OvoD1* mutation do not lay eggs but are still responsive to DR, indicating that DR can extend life span via a pathway(s) independent of those used for reproduction (Mair et al. 2004). This finding is supported by the fact that certain genetic interventions, such as overexpression of *dSir2*, can extend longevity via proposed DR-related mechanisms without decreasing female fecundity (Rogina and Helfand 2004).

5.5.4 Cognitive, Locomotor and Olfactory Functions

A number of studies have demonstrated that DR affects several *Drosophila* behaviors known to decline with age. As flies age they experience aging-related memory impairment (AMI) similar to higher organisms (Yamazaki et al. 2007). AMI is dependent on the activity of various *Drosophila* genes, including *amnesiac* (*amn*) and *discs overgrown* (*DCO*), that are active in neuronal structures critical for learning and memory, such as the Mushroom bodies. One study has shown that AMI, which typically emerges around 20 days of age in fully-fed flies, is significantly ameliorated when flies are subjected to DR (Yamazaki et al. 2007). The molecular mechanisms of DR effects on AMI remain to be determined. Experiments on other behaviors have failed to demonstrate a significant improvement in either locomotion or olfaction in response to DR (Bhandari et al. 2007). Other research however, has shown that nutritional status can affect activity levels in *Drosophila*, such that DR flies move more than fully-fed controls (Bross et al. 2005).

5.6 Molecular Mechanisms of DR

A major goal of DR research in *Drosophila* is to gain an understanding of the molecular pathways that regulate this biological phenomenon. It is hoped that this research will identify therapeutic targets for interventions designed to increase both the length and quality of human life. Two general strategies have been used to identify genes that mediate *Drosophila*'s response to DR: (1) identifying genes that when mutated block the prolongevity effect of DR, and, (2) subject long-lived flies caused by single gene modifications to DR. By the latter approach, if DR does not elicit a further extension in life span, then the gene is considered to be a DR gene.

5.6.1 The Insulin/IGF-1 Like Signaling Pathway

In rodents, lifespan extension by DR is associated with decreases of body size (Sonntag et al. 1999; Merry 2002; Masoro 2005) and circulating IGF-1 (and insulin) levels (Sohal and Weindruch 1996; Sonntag et al. 1999), suggesting that the insulin/IGF-1 like signaling (IIS) pathway lies at the interface between longevity, growth and nutrition. Regulation of the IIS pathway in the fruit fly by *Drosophila* insulin-like peptides (dILP) is nutrient sensitive, which makes the IIS pathway an obvious candidate mechanism for controlling the response to DR (Edgar 2006; Broughton and Partridge 2009). A possible role for the IIS pathway is also suggested by work focusing on the insulin receptor substrate *chico* (Clancy et al. 2001) (Fig. 5.2). Homozygous null mutations in *chico* generate flies that are long-lived and small (i.e., dwarf), two characteristics found in other model organisms (e.g., mice) in response to DR (Brown-Borg et al. 1996; Coschigano et al. 2000). Subsequent experiments revealed that *chico* null mutant flies are longer lived than controls

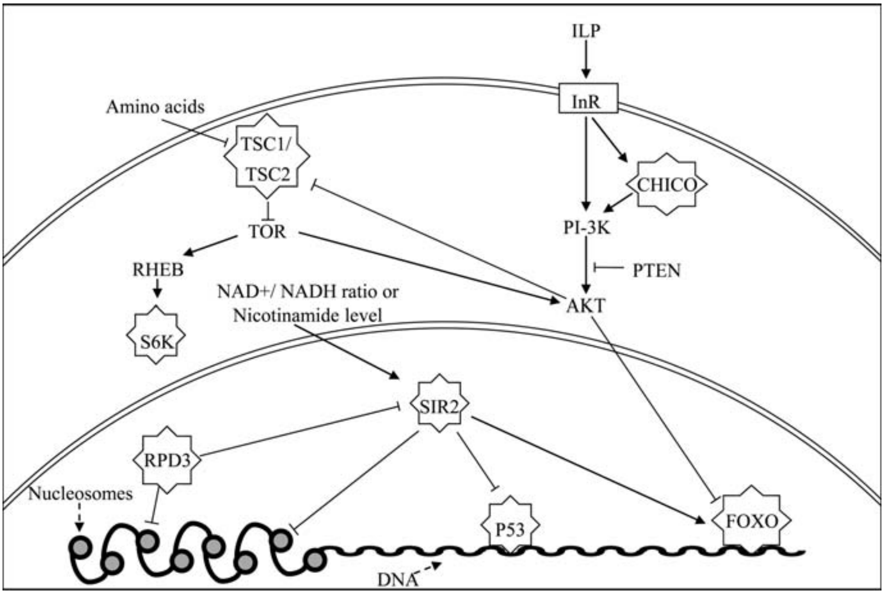

Fig. 5.2 Genetic pathways that are potentially involved in mediating the influence of DR on life span. Life span is extended by: (1) increases in the activity of the sirtuin pathway or (2) reductions in the activity of p53, the IIS pathway and the TOR pathway. The mechanism(s) used by these pathways to extend life span overlap, at least partially, with the mechanism(s) used by DR to influence *Drosophila* life span. Factors that have been tested in DR experiments are shown in stars

maintained on normal or high food concentrations, but shorter lived than controls on lower concentrations of food, appearing as though they are partially dietary restricted (Clancy et al. 2002). This suggests that DR and IIS regulate longevity through partially overlapping pathways.

Other evidence implies that the IIS pathway and DR influence longevity via distinct mechanisms (Giannakou et al. 2008; Min et al. 2008). These experiments have been done by either knocking down or overexpressing the *Drosophila* FOXO (*dFOXO*) gene, which encodes a forkhead transcription factor that is negatively regulated by the IIS pathway (Junger et al. 2003) (Fig. 5.2). When *dFOXO* is knocked-out, flies still respond normally to DR, suggesting that *dFOXO*, and hence IIS, is not required to regulate life span in response to nutrient conditions (Giannakou et al. 2008; Min et al. 2008). In contrast, another study reported that *dFOXO* overexpression in body fat yields a longevity profile similar to partial nutrient restriction (Giannakou et al. 2008), consistent with observations in *chico* null mutants (Clancy et al. 2002), and suggesting a role for *dFOXO* in the DR response. Other results challenge this view, showing that overexpression of *dFOXO* in *Drosophila* body fat significantly extends life span on all the diets tested (Min et al. 2008). A potential explanation of these discrepant results is that these arise from the different DR regimens employed, eliciting unique DR responses that operate through distinct signaling pathways. For example, in the nematode *Caenorhabditis elegans*, the IIS pathway is required for life span extension by one DR protocol that involves food dilution (*E. coli* on solid agar plates), but is not required for longevity extension induced by other DR methods (Lakowski and Hekimi 1998; Houthoofd et al. 2003; Kaeberlein et al. 2006; Lee et al. 2006; Greer et al. 2007). Alternatively, it may be that the IIS pathway is involved in the response to DR but is acting via a transcription factor other than *dFOXO*, a scenario that has recently been suggested in *C. elegans* (Panowski et al. 2007). Further complicating the picture is the fact that the IIS pathway is known to interact with other pathways, such as the Jun N-terminal kinase (JNK) pathway, Target of rapamycin (TOR) pathway, that influence the rate of aging (Gao and Pan 2001; Marygold and Leevers 2002; Wang et al. 2003; Kapahi et al. 2004; Wang et al. 2005; Edgar 2006). Whether a change in IIS is required for genotype-based mimicry of DR in *Drosophila* either alone or in concert with additional age-influencing pathways is still not known.

5.6.2 The TOR Pathway

The TOR pathway is involved in regulating cell growth in response to nutritional status (Edgar 2006). Using the UAS/GAL4 bipartite gene expression system (Brand and Perrimon 1993; Duffy 2002), researchers have shown that genetic interventions that decrease TOR signaling (e.g., overexpression of *dTsc2* or dominant negative *S6* kinase, upstream and downstream genes of TOR respectively) results in life span extension that cannot be enhanced through DR (Kapahi et al. 2004)

(Fig. 5.2). This implies that the TOR pathway and DR regulate longevity through a common mechanism. Furthermore, amino acid concentration, which is known to affect *Drosophila* longevity, affects the level of signaling through the TOR pathway, supporting a role for TOR signaling in modulating life span in response to nutrient levels (Gao and Pan 2001; Edgar 2006; Min and Tatar 2006b). Interestingly, evidence points to significant cross-talk between the IIS and TOR pathways, and it will be important to determine how nutrient uptake affects this interaction (Gao and Pan 2001; Marygold and Leevers 2002; Edgar 2006).

5.6.3 Apoptotic Pathways

p53, a tumor suppressor protein, is a key regulator of cellular fate. Based on the amount of genotoxic damage they sustain, p53 directs cells to either exit the cell cycle or undergo apoptosis (Das et al. 2008). A role for apoptosis in determining *Drosophila* life span has been proposed. During normal aging, the level of apoptosis increases in a tissue-specific manner, targeting the thoracic flight muscles, for example, and this increase is related to both physiological age and oxidative stress (Zheng et al. 2005a). Neuronal specific reduction of p53 activity, through expression of dominant negative forms of p53, results in an extension of life span in fully-fed controls, but not DR (Bauer et al. 2005) (Fig. 5.2). These results suggest that p53 regulates life span through a pathway also engaged in response to DR.

5.6.4 The Sirtuin Pathway

Sirtuins, a family of proteins with NAD+ dependent histone deacetylase activity, were first shown to be involved in the regulation of DR-mediated life span extension in experiments with the budding yeast *Saccharomyces cerevisiae* (Lin et al. 2000). In *S. cerevisiae*, replicative life span extension in response to caloric restriction, achieved by reducing the concentration of glucose from 2 to 0.5%, requires the activity of SIR2 (Silent Information Regulator), a founding member of the sirtuin family (Lin et al. 2000). In *Drosophila*, comparison of global gene expression in response to aging and DR indicates that many biological processes, including chromatin remodeling and histone deacetylation, are regulated by both aging and nutrient availability (Pletcher et al. 2002). Subsequent experiments in *Drosophila* confirmed, (1) the requirement of dSIR2 activity for life span extension in response to DR, and (2) that *dSir2* increases life span through a DR-sensitive pathway (Rogina and Helfand 2004) (Fig. 5.2). Interestingly, resveratrol, a compound with known sirtuin activating properties (Howitz et al. 2003), is believed to extend lifespan via the *dSir2* pathway in *Drosophila* (Wood et al. 2004). It should be noted, however, that the prolongevity influence of resveratrol has been challenged by a number of results demonstrating no drug effect on longevity in multiple species (Bass et al. 2007b; Kaeberlein and Kennedy 2007; Zou et al. 2009).

Of obvious interest to researchers is the identity of factors that both control and are controlled by *dSir2* and the role that these factors are playing in mediating the fruit fly's response to DR. DR has been shown to induce a significant decrease in the level of the histone deacetylase *Rpd3*, a factor believed to negatively regulate *dSir2* (Pletcher et al. 2002; Rogina et al. 2002). *rpd3* reduction-of-function mutations result in an increase in longevity via a pathway known to be responsive to food conditions (Rogina et al. 2002) (Fig. 5.2). Although the downstream targets of dSIR2 in DR response in *Drosophila* remain to be defined, mammalian SIRT1 has been shown to regulate the activity of both p53 and the mammalian FOXO factors, FOXO3A and FOXO4A (Luo et al. 2001; Vaziri et al. 2001; Giannakou and Partridge 2004; Bauer et al. 2005). SIRT1-mediated deacetylaton of p53 causes a decrease in its activity (Luo et al. 2001; Vaziri et al. 2001). In *Drosophila*, decreases in p53 activity result in life span extension via a pathway activated by DR (Bauer et al. 2005), suggesting the potential involvement of the apoptosis pathway mediated by *dSir2* and *dp53*.

5.6.5 The Olfactory System

Sensing the nutrient content of food is accomplished through both the olfactory and gustatory systems (Libert and Pletcher 2007). Experiments have shown that the presence of yeast odor is sufficient to decrease the life span of long-lived DR flies while having no effect on fully-fed controls (Libert et al. 2007). A genomic study of aging and DR flies has identified a number of genes encoding olfactory proteins whose expression is sensitive to DR. Genetic experiments have demonstrated that a mutation of one of these genes, *Odorant receptor 83b* (*Or83b* encoding a broadly expressed odorant receptor), increases life span, starvation resistance and oxidative stress resistance. Moreover, *or83b* mutant flies on DR still show increased longevity compared to wild type controls on DR, although the extent of increase in life span in fully-fed *or83b* mutant flies is relatively higher than that seen in DR *or83b* flies. Taken together, these results indicate that *Or83b* modulates life span partially through DR pathways (Libert et al. 2007).

5.7 Future Directions

A variety of theories of aging, such as the rate of living hypothesis and the oxidative stress theory of aging, have been employed to explain the mechanisms of DR-dependent life span extension. According to the rate of living theory, metabolic rate is a primary determinant of life span. Animals in a given species with relatively lower metabolic rates live longer than those with higher rates of metabolism. Intuitively, the decrease in food consumption that occurs during DR should result in a lowered metabolic rate and extended life span. It has been proposed that a lowered metabolic rate would decrease the production of ROS through oxidative respiration.

According to the oxidative stress theory of aging, DR increases lifespan through a reduction of ROS production and/or an increase of ROS scavenging. Experimental data from multiple laboratories contradict many of the predictions of these theories. Contrary to the rate of living hypothesis, DR does not result in a change in the rate of resting metabolism. Whether DR alters the metabolic rate of active flies remains to be determined and may help clarify the role of metabolic rate in the DR response. Contrary to the oxidative stress theory, available evidence fails to reveal any DR-dependent reduction in the level of ROS or oxidative stress experienced by DR flies. However, DR-dependent changes in mitochondrial morphology and the level of oxidatively-derived lipid damage have been observed, suggesting that DR may influence the oxidative status of the fly.

An alternative framework for understanding the influence of diet on life span focuses on hormesis. Hormesis can be defined as a mild-stress induced enhancement of protective mechanisms that are biologically beneficial to organismal health (Rattan 2008). By this account, by mildly increasing the levels of specific biological stressors (e.g., induced via ROS), DR can elicit protective responses that enable better coping when confronted with these and other stressors later in life (Masoro 2007; Rattan 2008). It seems unlikely that one of these theories alone fully accounts for DR-dependent longevity extension. Further experimental work is required to clarify the contributions of metabolic, oxidative stress and hormesis mechanisms to the interaction between diet and life span.

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Chapter 6
Aging and Dietary Restriction: The Yeast Paradigm

Min Wei, Federica Madia, Rafael de Cabo,
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6.1 Introduction

Seventy years after the first demonstration of life span extension by dietary restriction (DR) (McCay et al. 1935), the mechanism underlying its beneficial effect remains only partially understood (Bergamini et al. 2003; Anderson et al. 2009). Owning to their short life span and amenability to genetic and experimental manipulations as well as the conserved signaling and basic cellular processes among eukaryotes, unicellular yeast, invertebrate animals, worm and flies, and rodents are widely studied to understand the mechanisms of aging and diseases. DR regimen ranging from glucose or amino acid limitation and incubation in water for yeast, axenic medium or bacterial deprivation for worms, reduced availability of yeast for flies, calories or amino acid restriction or alternate day feeding for rodents, lead to a 20% to more than 100% life span extension in model organisms (Orentreich et al. 1993; Jiang et al. 2000; Lin et al. 2000; Bartke et al. 2001; Houthoofd et al. 2002; Mattson 2005; Partridge et al. 2005; Powers et al. 2006; Sutphin and Kaeberlein 2008; Wei et al. 2008). The effects of these dietary restricted regimens on life span have been linked to the evolutionary advantage provided by entering into alternate states that can maximize survival through long periods of famine while minimizing any negative effect on the level and quality of reproduction once the food becomes available (Longo and Finch 2003; Brunet 2009).

Aging research in *Saccharomyces cerevisiae* essentially begin with the finding that yeast mother cells undergo a finite number of divisions (Mortimer and Johnston 1959; Muller et al. 1980; Pohley 1987; Jazwinski et al. 1989), which is now termed replicative (budding) life span (RLS). Asymmetrical division of the budding yeast results in the accumulation, in the mother cells, of extrachromosomal ribosomal DNA circles (ERCs), oxidized proteins and damaged organelles, which contribute to replicative senescence, permanent cell-cycle arrest, and, eventually, cell lysis

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(Mortimer and Johnston 1959; Muller et al. 1980; Jazwinski et al. 1989; Kennedy et al. 1994; Sinclair and Guarente 1997; Lai et al. 2002; Aguilaniu et al. 2003; Jazwinski 2005; Nystrom 2005; Seo et al. 2007).

The other paradigm to study yeast aging is the chronological life span (CLS), which measures the chronological survival of non-dividing yeast in culture (Longo et al. 1996; Fabrizio et al. 2001; Fabrizio and Longo 2003). Yeast replicative aging has been considered a model for cellular senescence in mammals since certain mammalian cells also undergo a limited number of population doublings in culture. By contrast, the yeast chronological survival models the aging of post-mitotic cells in higher eukaryotes but also provides a simple model for organismal aging (Fabrizio and Longo 2003; Longo and Kennedy 2006). Studies using the two aging models of yeast have uncovered distinct but also overlapping life span regulatory pathways that appear to be partially conserved in higher eukaryotes (Sinclair et al. 1998; Fabrizio et al. 2003; Longo and Finch 2003; Kenyon 2005; Piper et al. 2006; Steinkraus et al. 2008).

6.2 DR in Replicative Life Span Studies

Dietary manipulation by reducing the concentration of glucose in rich medium (YP) from the standard 2 to 0.5% or 0.005% lengthens yeast replicative life span (RLS)(Lin et al. 2000; Kaeberlein et al. 2004; Kaeberlein et al. 2005a; Smith et al. 2007). Lowering the non-essential amino acids and/or the nitrogen availability while maintaining glucose level in the medium also extends yeast RLS, suggesting that the reduction of calories, but not of one particular nutrient, promotes longevity (Jiang et al. 2000). Glucose limitation-based DR has been reported to inhibit the formation of ERCs in the nucleolus (Kaeberlein et al. 1999), and to increase NAD/NADH ratio or reduce nicotinamide (Lin et al. 2004; Lamming et al. 2005; Sinclair 2005). DR also increases respiration and boosts mitochondrial functions, decreases proton leakage and ROS production in the mitochondria (Lin et al. 2002; Barros et al. 2004; Pamplona et al. 2004; Sanz et al. 2006), and attenuates the accumulation of oxidative damage (Reverter-Branchat et al. 2004).

6.3 DR in Chronological Life Span Studies

After approximately 10 h of exponential growth in glucose-containing liquid synthetic complete (SDC) medium, yeast cells switch from fermentation to respiration utilizing non-fermentable carbon sources such as ethanol and lactate generated during fermentation, a phase termed diauxic shift. Population growth continues slowly in the following post-diauxic phase for another 24–48 h. Cells display high metabolic rate for 4–5 days and then begin to die rapidly (Fabrizio et al. 2003). Growing yeast in glucose-limited medium (0.5 or 0.05% glucose) or switching post-diauxic/stationary phase cells to water, both of which model nutrient-depleted conditions yeast may encounter in the wild, also promote chronological survival (Longo 1997; Fabrizio et al. 2005; Smith et al. 2007; Wei et al. 2008). DR in the CLS paradigm has been reported, as in replicative studies, to increase cellular protection against heat and oxidative stresses, to prevent protein oxidative damage, reduce the level of iron and of lipid peroxidation, and enhance oxidative stress response through maintenance of high levels of catalase (Ctt1) and superoxide dismutase enzymes (Sod1, Sod2) (Reverter-Branchat et al. 2004). In contrast with the replicative life span model, ERCs do not seem to play a role in chronological aging of cells (Ashrafi et al. 1999).

6.4 Genetic Mediators of DR

Various mutants with deficiencies in glucose-sensing were used as genetic models of DR. Two major pathways, the Ras/cAMP/PKA and Snf3/Rgt2, transduce glucose signaling (Santangelo 2006). For the PKA pathway, G proteins (Ras and Gpa2) activate the adenylate cyclase (Cyr1) to produce cAMP. Binding of cAMP to Bcy1, the regulatory subunit of PKA, releases and activates the PKA kinase subunits (Tpk1, 2 and 3). Ras activity is regulated positively by the guanine nucleotide exchange factors (RasGEFs, Cdc25 and Sdc25) and negatively by the GTPase-activating proteins (RasGAPs, Ira1 and Ira2). Deletion of *Cyr1*, *CDC25*, or the PKA catalytic subunits extend, whereas deletion of gene that dampens PKA signaling shortens, yeast RLS (Lin et al. 2000). Although the plasma membrane glucose sensors Snf3 has not been linked to life span regulation, deletion of *HXK2*, the first glycolytic enzyme that phosphorylates glucose and functions as glucose-responsive transcriptional repressor, extends yeast RLS (Lin et al. 2000; 2002; Kaeberlein et al. 2005; Lamming et al. 2005). Similarly, mutants with deficiencies in the Ras/Cyr1/cAMP/PKA signaling pathway and those lacking both HXK2 and MIG1, a transcription factor involved in glucose repression, have extended chronological life span (Longo 1997; Fabrizio et al. 2001; Lorenz et al. 2009).

The partially-conserved Ras/cAMP/PKA and Tor/Sch9 signaling pathways integrate the nutrient and other environmental cues to regulate cell growth and division in yeast (Jorgensen et al. 2004; Martin et al. 2004; Santangelo 2006). Mutations dampening TOR (specifically the TOR Complex I activity) signaling, a key nitrogen-sensing pathway that controls protein synthesis and degradation (Lorberg and Hall 2004), and the downstream S6 kinase/AKT homolog *SCH9* (Geyskens et al. 2000; Urban et al. 2007) have also been shown to lengthen yeast life span (Fabrizio et al. 2001; Kaeberlein and Kennedy 2005; Powers et al. 2006; Wei et al. 2008). Genetic evidence suggests that the mechanisms underlying the effect of DR on life span involve both the Tor/Sch9 and Ras/cAMP/PKA pathways. In fact, glucose limitation (0.5 or 0.05% vs. the standard 2% glucose) fails to further extend the RLS of *cdc25-10*, *sch9*Δ or *tor1*Δ mutants, indicating that DR by glucose limitation is mediated by down-regulation of the cAMP/PKA, Tor and Sch9 pathways (Lin et al. 2000; Kaeberlein et al. 2005c; Powers et al. 2006; Medvedik et al. 2007). For chronological survival, glucose limitation- and extreme CR/starvation (water)-induced life span extension requires the protein kinase Rim15 and its downstream stress response transcription factors: Msn2/4 and Gis1, which are negatively regulated by PKA, Tor and Sch9 (Vidan and Mitchell 1997; Roosen et al. 2005; Swinnen et al. 2006). Whereas DR-induced CLS extension is abolished by the deficiency in Rim15, it is only partially reversed by triple deletion of *MSN2/4* and *GIS1*, suggesting the existence of additional transcription factor(s) or mediator(s) downstream of Rim15 (Wei et al. 2008). Incubation of aging cells in water further extends CLS of *tor1*Δ, *sch9*Δ and *ras2*Δ mutants (Longo 1997; Fabrizio et al. 2005; Wei et al. 2008) and leads to a 10-fold lifespan extension when combined with *sch9*Δ *ras2*Δ double deletion, which only partially depends on Rim15 (Wei et al. 2008). Taken together, the data suggest that down-regulation of the Ras/cAMP/PKA and Tor/Sch9 pathways and up-regulation of Rim15 and transcription factors Msn2/4 and Gis1 account for a major portion but not all the beneficial effects of DR/starvation on life span (Fig. 6.1).

Autophagy is an intracellular process aimed at the degradation of damaged proteins and organelles, and its deficiency has been connected to accelerated aging in various species from yeast, *C. elegans*, flies to mice (Melendez et al. 2003; Kuma

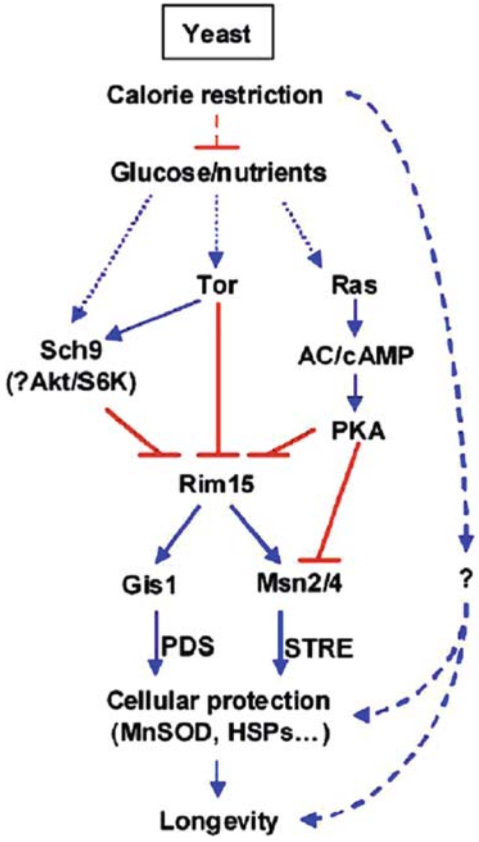

Fig. 6.1 Cellular pathways for the effects of caloric restriction on longevity in yeast

et al. 2004; Juhasz et al. 2007). Interestingly, autophagy is activated in response to different stressors and is sensitive to nutrient availability (Kuma et al. 2004; Mizushima et al. 2008). In yeast, autophagy may be involved in the replicative life extension caused by *TOR1* deletion (Kaeberlein et al. 2005c). Three yeast autophagy-related proteins have been identified as substrates of PKA (Budovskaya et al. 2005). Furthermore, a recent study in yeast has implicated Sch9 to be a negative regulator of autophagy in a Rim15/Msn2/4-dependent manner (Yorimitsu et al. 2007). However, the link between dietary restriction, longevity and autophagy has not been fully characterized. It is possible that DR might induce this process by down-regulating nutrient responsive pathways, decreasing protein synthesis and triggering other metabolic changes (Yen and Klionsky 2008).

6.5 Good, Bad, and Neutral Carbs, Lessons from Long-Lived Genetic Mutants

Using a plate CLS assay, which resembles the procedure employed in RLS studies, where cells were exposed to various types and concentrations of nutrients but not allowed to divide (Madia et al. 2007; Wei et al. 2008), we demonstrated that the presence of amino acids and nitrogen-bases shortens the viability of yeast compared to cells plated on agar (water). Addition of ethanol, and worse glucose, exacerbates cell death (Wei et al. 2008). Although glucose is usually depleted by day 1 after cells enter diauxic shift, ethanol level remains high in wild type culture until day 7 when approximately 50% cells are still alive. By contrast, cells deficient in Sch9 and, to a less extent, those deficient of Tor1 or Ras2 deplete the ethanol by day 3, suggesting that removing pro-aging carbon source(s) contributes the extended CLS in long-lived genetic mutants. Indeed, the global gene expression profiling of these mutants revealed that glycerol biosynthesis genes, along with glucose utilization genes (glucose transporters and glycolytic enzymes), are upregulated in all three mutants. Genetic analysis further confirmed that not only the expression of glycerol genes are under the control of Gis1, a stress response transcription factor negatively regulated by Sch9, Tor and Ras, but they are required for the stress resistance and longevity promoting effects in cells lacking Sch9 (Wei et al. 2009).

Glycerol is a byproduct of the overflow metabolism when there is enhanced glycolytic flux and limited respiration capacity in yeast (Crabtree 1928; van Dijken et al. 1986). It has long been known to protect cells from heat, osmotic and oxidative stresses by acting as a chemical chaperon, by retaining water as well as through its role in the maintenance of NAD^+:NADH ratio (Albertyn et al. 1994; Ansell et al. 1997; Bakker et al. 2001; Meng et al. 2001; Wojda et al. 2003; Rigoulet et al. 2004; Deocaris et al. 2006). Although both high osmolarity and NADH shuttle components have been linked to life span extension in yeast (Kaeberlein et al. 2002; Easlon et al. 2008; Murakami et al. 2008), addition of glycerol to wild type yeast grown in standard medium or increase NADH oxidation by overexpressing NADH oxidase did not lead to significant change of life span (Wei et al. 2009) (unpublished results). In contrast with glucose and ethanol, presence of either carbon source promotes aging, glycerol not only does not shorten life span of DR-ed yeast, its uptake and utilization may contribute to long-term survival (Wei et al. 2008). Taken together, the data suggest the genetically induced "carbon source substitution" of the pro-aging ethanol with the neutral glycerol in long-lived mutants creates a DR-like environment.

6.6 Sirtuins and DR

The Sir2 family of class III histone deacetylases is characterized by the requirement of nicotinamide adenine dinucleotide (NAD^+) as cofactor for its enzymatic activity (Denu 2003). Two non-exclusive models have been proposed linking metabolism to Sir2 activity in yeast: DR by glucose limitation activates Sir2 by increasing NAD^+/NADH ratio (Lin et al. 2002) and depleting the Sir2-inhibitory nicotinamide by increasing the expression of PNC1, an enzyme that converts nicotinamide to nicotinic acid via the stress response transcription factors Msn2 and Msn4 (Bitterman et al. 2002; Anderson et al. 2003; Medvedik et al. 2007). The finding that Sir2 mediates the beneficial effects of DR also spawned the search for small molecules that activate Sir2 and its homologs in higher eukaryotes that may function as DR mimetics, among which the polyphenol compound resveratrol was shown to extend yeast RLS by 70% (Howitz et al. 2003; Milne et al. 2007; Pearson et al. 2008).

The mechanisms underlying the positive effect of increased dose of Sir2 and sirtuin activating compounds, however, are still not well understood. In yeast, the role of Sir2 regulating life span seems to be strain specific, which may be partly explained by the existence of redundant homologous deacetylases; depending on the extent of glucose reduction, DR seems to exert its pro-longevity effects through Sir2-dependent and -independent pathways (Kaeberlein et al. 1999; Lin et al. 2000; 2002; Kaeberlein et al. 2004; Kaeberlein et al. 2005b; Lamming et al. 2005; Tsuchiya et al. 2006). In contrast with the findings in replicative life span studies, using single-gene deletion mutants, extension of chronological survival by DR does not require Sir2 or the other NAD^+-dependent histone deacetylases (Hst1, 2, 3, or 4), although the possibility of redundancy between the sirtuins was not tested (Smith et al. 2007). Moreover, deletion of the *SIR2* gene further extends the life span of already long-lived mutants lacking Sch9 or components of the Ras/cAMP/PKA pathway (Ras2 and Cyr1) as well as of yeast undergoing extreme DR/starvation (Fabrizio et al. 2005 and Wei and Madia, unpublished results). Resistance to heat and oxidative stresses, a phenotype often associated with longevity, is also enhanced in the *sir2*Δ mutants supporting the pro-aging role of Sir2 in increased stress sensitivity and blocking extreme chronological longevity in yeast (Fabrizio et al. 2005). A similar observation was also made in *C. elegans* where resveratrol extended life span by activating ER stress response genes that are repressed by worm *SIR2* ortholog (Viswanathan et al. 2005). Although the link between sirtuins and aging is complex and paradoxical (Kaeberlein et al. 2005b; Longo and Kennedy 2006; Longo 2009), accumulating evidence points to the involvement of sirtuins in myriad of cellular processes, whose dysregulation and dysfunction leads to age-associated diseases (Chen and Guarente 2007) and warrant in-depth studies of the role of sirtuins in enhancing organismal health span.

6.7 Superoxide, Mitochondria and Genomic Stability

One of the major causes of aging in aerobic organisms is the accumulation over time of macromolecular damage induced by reactive oxygen species (ROS) (Harman 1956). Oxidative damage to macromolecules has been demonstrated in both replicative and chronological life span paradigms (Reverter-Branchat et al. 2004). Mutations in the Ras/cAMP/PKA pathways increase survival and resistance to oxidative stress (Longo 1999; Fabrizio et al. 2001). Overexpression of the constitutively active *RAS2*$^{\mathrm{val19}}$ leads to reduced replicative and chronological life span, increased production of ROS, and elevated oxidative protein damage (Longo 1999; Hlavata et al. 2003; Wei et al. 2009). Knockout strains lacking either the cytosolic Cu/ZnSOD (*SOD1*) or the mitochondrial MnSOD (*SOD2*) exhibit reduced growth rates on respiratory carbon sources, amino acid auxotrophies and shortened chronological and replicative life span (Longo et al. 1996; Longo 1999; Fabrizio et al. 2003; Harris et al. 2003; Unlu and Koc 2007). Sod2 is also required for the life span extension in the cells with deficiencies in Sch9 or Ras/cAMP/PKA signaling. However, its role is necessary, but not sufficient, since the CLS extension by *SOD2* overexpression is much less prominent compared to that of *SCH9*, *RAS2*, or *CYR1* deletion (Fabrizio et al. 2003). The *sch9*Δ mutants show reduced age-dependent inactivation of the superoxide-sensitive enzyme aconitase (Fabrizio et al. 2001), higher expression of the mitochondrial MnSOD (Fabrizio et al. 2003) and reduced cellular superoxide levels, indicating that enhanced protection against and/or decreased production of mitochondrial ROS promotes CLS. By contrast, lack of cytosolic Sod1, but not mitochondrial Sod2, reduced yeast RLS (Kaeberlein et al. 2005b), which may reflect the factor that yeast cells derive most of the energy through substrate-level phosphorylation in the RLS analysis.

In yeast, DR induces the expression of *SOD1*, *SOD2*, catalase and glutathione peroxidase (GPx) (Agarwal et al. 2005) and decreases oxidative damage to stress resistance proteins and key enzymes involved in glucose metabolism (Reverter-Branchat et al. 2004). The role of mitochondrial function and respiration in DR-induced life span extension is still unclear. Loss of mitochondrial DNA (the respiration-deficient petite) extends yeast RLS, which is initially shown to be due to the activation of retrograde response, an altered nuclear gene expression in response to changes in mitochondria, and its inhibition of ERCs formation (Kirchman et al. 1999; Borghouts et al. 2004). However, new results suggest that the strength of retrograde regulation does not correlate with life span (Woo and Poyton 2009). Glucose-limiting DR is proposed to extend yeast RLS by increasing respiration (Lin et al. 2002). However, the life span of petites can be further lengthened by glucose limitation (Kaeberlein et al. 2005a), suggesting respiration is not required for the

DR-induced RLS extension. For chronologically-aging yeast, pre-adaptation to efficient respiratory maintenance ensures maximal CLS (Piper et al. 2006). Studies have also shown that increased respiration is required for increasing CLS in the *sch9*Δ and *tor1*Δ mutants (Bonawitz et al. 2007; Lavoie and Whiteway 2008). Furthermore, depletion of the pro-aging carbon source ethanol by respiration also contributes to life span extension in *sch9*Δ mutants (Fabrizio et al. 2005; Wei et al. 2009).

In a CLS model, starvation/extreme DR (switch to water) showed a promising protective trend but not a significant effect against age-dependent mutation frequency in premature aging caused by deficiency of Sgs1, a helicase homolog of mammalian Blm and Wrn (Madia et al. 2008). *sgs1Δ* mutants have been also reported to induce ERCs and premature aging in a RLS model (Sinclair and Guarente 1997). Mutations in *SCH9*, but not DR, prevented the occurrence of spontaneous and induced age-related nuclear mutations (Fabrizio et al. 2005; Madia et al. 2008) (unpublised data), prevented recombination errors and prevented the premature genomic instability caused by Sgs1 helicase deficiency. The effect of Sch9 on mutations might be linked to the regulation of antioxidant enzymes through its downstream effectors and or the generation of ROS via a error-prone repair mechanism (Longo et al. 2008; Madia 2009).

6.8 Conclusion

In the past 10 years, great progress has been made to elucidate the mechanisms underlying and to identify the targets that mediate the anti-aging effect of dietary restriction in yeast. Studies using the two aging models of yeast have uncovered distinct but overlapping life span regulatory pathways that appear to be partially conserved in higher eukaryotes. Although competing theories remain to be proven and key questions to be answered, findings originated from this simple unicellular organism indicate that the down-regulation of nutrient signaling pathways and up-regulation of stress resistance systems that are partially conserved from yeast to mammals play key roles in the effects of DR on aging and resistance to endogenous and exogenous toxins.

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Chapter 7
The Nutritional Geometry of Aging

Stephen J. Simpson and David Raubenheimer

7.1 Introduction

Dietary restriction (DR) without malnutrition prolongs life in an extraordinarily diverse range of organisms. Although it is generally believed that increased longevity with dietary restriction arises from eating fewer calories (termed caloric restriction, CR), there is an increasing body of evidence indicating that restriction of certain nutrients, rather than calories per se, is responsible for increased life expectancy (Zimmerman et al. 2003; Mair et al. 2005; Piper et al. 2005; Ayala et al. 2007; Hulbert et al. 2007; Carey et al. 2008; Hulbert 2008; Lee et al. 2008; Maklakov et al. 2008; Dussutour and Simpson 2009).

Separating the effects of nutrients and calories is not straightforward. Recently (Simpson and Raubenheimer 2007) we argued that the state-space modelling platform, known as the Geometric Framework (GF) (Raubenheimer and Simpson 1993; Simpson and Raubenheimer 1993; Raubenheimer and Simpson 1997; Simpson et al. 2004) provides a new tool for disentangling the influences of calories and nutrients. Since the publication of that paper, three studies on insects (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009) have employed the GF and demonstrated that caloric restriction is not responsible for the life extending benefits of DR; rather the protein/carbohydrate balance is implicated as the key dietary factor. In this chapter we begin by explaining the GF, then summarize the results of the recent experiments which have used this approach. Finally, we discuss how these insect experiments can help in the design of experiments for mammalian systems.

7.2 The Geometric Framework

The GF is a set of graphical models which originated from studies of insects and has since been used on other taxa (invertebrates, mammals, birds and fish) in various

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contexts, including the dietary causes of obesity (Simpson and Raubenheimer 2005; Sørensen et al. 2008), food animal production (Ruohonen et al. 2007) and the conservation of endangered species (Raubenheimer and Simpson 2006; Felton et al. 2009). In brief, the GF considers the simultaneous and changing requirements of an organism for multiple nutrients, the relative values of foods in relation to these requirements, the behavioral and post-ingestive responses of animals on diets of varying composition, and the physiological and performance consequences of being confined to particular dietary regimes.

In the GF the current and ideal nutritional states of an organism are represented either as points (intake, metabolic and growth "targets") within multidimensional nutrient space integrated over a particular time period, or else as trajectories moving in time. Within the same nutrient space, foods are represented as vectors called nutritional rails. As the animal eats, its nutritional state changes along the same vector as that of the food. A nutritionally balanced food will allow the animal to reach its intake target, and thus to achieve its requirements for all nutrients simultaneously, whereas nutritionally imbalanced foods force a compromise between over-ingesting some nutrients and under-ingesting others, with associated costs. Complementary imbalanced foods allow an animal to mix an optimal diet by switching between foods, using excess nutrients ingested from one food to redress the deficits incurred on the other. When neither nutritionally balanced nor complementary foods are available the animal cannot balance its nutrient intake, but it can selectively void ingested excesses post-ingestively by excretion or wastage metabolism [e.g. facultative diet-induced thermogenesis (Zanotto et al. 1997; Stock 1999)] and thus regulate to some degree body composition and growth. When such post-ingestive regulatory systems reach their limits, the animal will suffer the costs of ingested excesses of some nutrients and/or deficits of others. Thus, for example, ingesting excess non-protein energy to maintain protein intake results in obesity in humans, rodents and insects (Raubenheimer et al. 2005; Simpson and Raubenheimer 2005; Warbrick-Smith et al. 2006; Sørensen et al. 2008). That both ingested excesses and deficits of nutrients incur costs indicates that concepts such as "hormesis" apply equally to nutrition as to toxicology (Raubenheimer and Simpson 2009).

7.3 Geometric Experimental Designs for Aging Research

7.3.1 Which Dimensions to Include in Models?

When considering the relationship between the effects of nutrients and calories, initial experimental designs should include the major sources of calories as focal dimensions. In most cases, this will mean the macronutrients carbohydrate, fat and protein. Depending on the results of such experiments, these axes can then be disaggregated in further designs to consider the individual and interactive effects of component amino acids, fatty acids or carbohydrates.

As an aside to the main theme of calories vs. nutrients, it is worth noting here that other components of food such as plant secondary metabolites play important roles in food selection, ingestion and nutrient utilization and some are strongly implicated in aging (Baur et al. 2006; Kanfi et al. 2008). When considering their possible effects on aging, such chemicals should be accommodated as dimensions along with nutrients, to allow exploration of their pre- and post-ingestive influences and nutritional interactions: the nutritional context may well be critical to understanding the effects of a given dietary additive (Simpson and Raubenheimer 2001; Raubenheimer and Simpson 2009).

7.3.2 Deriving Nutrient Intake Arrays

Having chosen the key dimensions, the aim is to generate a range of intake points in nutrient (and, if relevant, other dietary component) space, upon which response surfaces related to longevity and markers of aging can be constructed. In the case of the insect experiments described below, protein and carbohydrate (the two major macronutrients for these species) provided tractable two-dimensional surfaces and the array of intake points on this plane was generated by restricting animals to one of a large number of diet compositions, varying in the ratio of protein to carbohydrate (P:C) and in the concentration of these nutrients within a given P:C ratio. By allowing ad libitum access to food rather than offering a fixed food ration, animals were allowed the opportunity to express compensatory feeding within a P:C ratio for nutrient concentration. It is important to measure the amount of food actually eaten under such circumstances (something which is not done in most experiments on invertebrates), since failing to take account of compensatory feeding and constructing response surfaces based on diet composition rather than actual nutrient intake risks serious misinterpretation. Despite recent commentary among *Drosophila* workers (Wong et al. 2008), evidence from a wide range of invertebrates, including various species of flies, indicates that compensatory feeding is to be expected (e.g. Simpson et al. 1989; Simpson and Simpson 1990; Raubenheimer and Simpson 1993; Cruz-Rivera and Hay 2000; Lee et al. 2004). The extent of compensatory feeding differs between species and depends upon the level of dietary dilution and the ratio of nutrients in the diet. It is important to measure food intake over prolonged periods rather than attempting to extrapolate from short-term assays, to avoid confusing short-term sensory responses from long-term regulation (Simpson et al. 1989; Dussutour and Simpson 2008).

7.3.3 Mapping Response Surfaces onto Intake Arrays

Having derived an array of nutrient intake points, response surfaces can be constructed on these arrays and compared. The comparison of such surfaces between species and genotypes within species offers considerable promise in the search for common patterns and associated mechanisms. The primary response variables will include maximum and median life expectancy, rate of mortality, delay to the onset of maximum mortality, and perhaps changing susceptibility to mortality factors such as disease. Response surfaces can also be constructed for various cellular, molecular and biochemical processes that are considered to reflect aging and might be mechanistically linked to the biology of aging, including mitochondrial metabolism, oxidative stressors, measures of immune function, telomere length, advanced glycation end products, histone deacetylases (e.g. sirtuins), cell membrane fatty acid composition, hepatic microcirculation (in mammals), and so forth (Butler and Sprott 2000; Le Couteur et al. 2002; McLean and Le Couteur 2004; Bordone and Guarente 2005; Guarente and Picard 2005; Hulbert et al. 2007; Hulbert 2008). GF designs offer a powerful tool for exploring the genetics of aging, initially by plotting response-surfaces for patterns of gene expression onto nutrient intake arrays. Additionally, the opportunity exists to compare measures of lifespan with other life-history traits, such as reproductive effort, to establish the nature of nutritional covariances and possible causal relationships (Lee et al. 2008; Maklakov et al. 2008).

7.3.4 How Does Regulation of Nutrient Intake Map onto Response Surfaces?

It has been demonstrated using the GF that animals from a range of taxa have the capacity to regulate their intake of specific nutrients, notably protein and non-protein energy (Raubenheimer and Simpson 1997; Mayntz et al. 2005, 2009; Lee et al. 2008; Sørensen et al. 2008). In conjunction with experiments in which animals are confined to a single diet, it is instructive to allow animals to self-select between pairs of nutritionally complementary foods to allow the mechanisms regulating intake of different nutrients to be expressed. The aim is to discover to which point (integrated over a given time period) or trajectory (followed over time) animals tend towards in nutrient intake space, how well they regulate when challenged with different pairs of complementary foods, and how the regulated intake point/trajectory maps onto the various response surfaces from no-choice experiments.

7.3.5 Availability and Allocation of Ingested Nutrients

The GF provides a means of quantifying and interpreting the availability and allocation of ingested nutrients (not just energy) to growth, storage and metabolism (Raubenheimer and Simpson 1994; 1995; Raubenheimer et al. 2009). Interactions among nutrients once ingested can be complex and subtle. For example, methionine restriction extends lifespan in rodents (Zimmerman et al. 2003) but the associated depression in plasma glucose and insulin concentrations

(Miller et al. 2005) might indicate that the effect of methionine restriction is mediated indirectly through impaired carbohydrate utilisation and thus energy restriction.

7.4 Three Examples of Geometric Designs Using Insects

The first study to employ a GF design in relation to lifespan was that of Lee et al. (2008) using *Drosophila*. Mated female flies were confined singly to one of 28 diets, varying in the ratio and concentration of yeast to sugar. Intake was quantified volumetrically (Ja et al. 2007; Simpson and Raubenheimer 2007) and surfaces for lifespan, the age of maximal mortality, the rate of age-dependent increase in mortality, lifetime egg production and rate of egg production were mapped onto points of intake on a protein–carbohydrate plane. Two of these surfaces (lifespan and lifetime egg production) are shown in Fig. 7.1. The longevity plot shows unequivocally that CR was not responsible for extended lifespan. Flies lived longest on a diet containing a 1:16 P:C ratio and lived progressively less long as the P:C ratio increased. The contours of the longevity surface run almost orthogonally to lines of equal caloric intake (dotted lines in Fig. 7.1). Even allowing for possible differences in the relative availability of energy in protein and carbohydrate or interactions between protein and carbohydrate metabolism (see above), there is no way that CR could account for the variation in lifespan (i.e. that the lifespan and caloric intake isoclines in Fig. 7.1 can be aligned). Rather, the balance of carbohydrate to protein (or some associated constituent of yeast) was responsible (see below).

The response surface for lifetime egg production reached a maximum at a higher protein content than supported maximal lifespan (1:4 P:C), whereas rate of egg production was maximal at 1:2 P:C. When flies were offered one of 9 complementary food choices (separate yeast and sugar solutions differing in concentration) they converged upon a trajectory of 1:4 P:C, thereby maximising lifetime egg production. Compensatory feeding responses were marked, but incomplete on some food combinations. Thus if sugar was presented at 180 g/L flies maintained intake of yeast constant across a fourfold dilution range (45–180 g/L), but when the sugar solution was more dilute, some of the limiting carbohydrate was gained by eating extra yeast solution, with the consequent increased intake of protein being associated with reduced longevity.

Lee et al. (2008) compared their data against a longevity surface compiled from previously published studies, individually involving many fewer dietary treatments and no measurement of long-term food intake. The two surfaces corresponded closely, despite substantial procedural differences across studies and differences in mean lifespan between capillary-fed, singly housed flies in the study of Lee et al. (2008) and flies housed in groups and fed agar-based diets in the other experiments. To further demonstrate that the nutritional associations were robust, traditional demography cage trials were run for a selection of diets without measuring intake. Flies lived longer than when housed singly and fed from capillaries, but the pattern

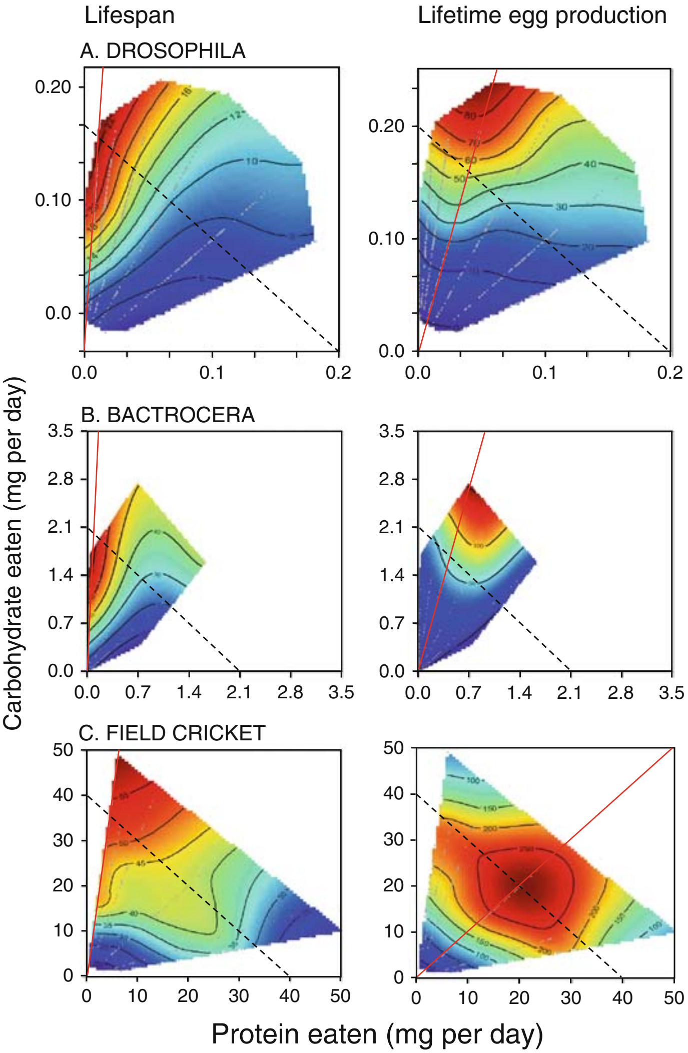

Fig. 7.1 Response surfaces for longevity and lifetime egg production in three species of insect provided with ad libitum access to one of 28 (*Drosophila* and the Queensland fruit fly, *Bactrocera*) or 24 (field cricket) diets varying in the ratio and total concentration of protein to carbohydrate (P:C) throughout their life. Surfaces rise in elevation from *dark blue* to *dark red* and are plotted onto arrays of nutrient intake. *Red lines* indicate the dietary P:C that maximised the response variable, whereas the *dotted lines* indicate isocaloric intakes. In each case, insects lived longest when the diet contained a low P:C ratio, with lifespan declining as P:C rose. Caloric effects alone cannot have caused this pattern. In contrast, female reproductive performance was maximal on higher P:C diets, but excess protein intake beyond this optimum resulted in a decline in reproductive success even at high total energy intakes. Data are from Lee et al. (2008) (*Drosophila*), Maklakov et al. (2008) (field crickets) and Fanson et al. 2009) (*Bactrocera*)

of lifespan, egg production and egg production rate in relation to dietary P:C ratio was the same.

A parallel study to that of Lee et al. (2008) was run by Fanson et al. (2009), using a tephritid; the Queensland fruit fly, *Bactrocera tryoni*. There were 28 no-choice diet treatments and 25 choice combinations. Results and conclusions were similar in all respects to those reported by Lee et al. (2008) (see Fig. 7.1). The data were compared directly with the study of Carey et al. (2008) on another tephritid, *Anastrepha ludens*, in which intake was not measured. There was strong concordance between the two data sets. The importance of measuring intake was indicated by a comparison of surfaces derived from dietary composition alone and those constructed using actual nutrient intakes. Response surfaces that ignored compensatory feeding (i.e. based on diet composition not actual intake) seemed to show an increase in lifespan due to CR, especially at low P:C ratios. When actual nutrient intake was used, however, there was no life-extending effect of CR. Rather, CR caused a decline in lifespan at higher P:C ratios.

In the studies of Lee et al. (2008) and Fanson et al. (2009) longevity was primarily associated with the ratio of yeast to sugar eaten, not calorie intake. Yeast, however, is a complex food, containing various micronutrients in addition to protein and carbohydrate. A recent paper on *Drosophila* (Troen et al. 2007) used four chemically defined diets in which methionine and glucose were varied. Small but significant effects of dietary methionine on lifespan were reported (egg production was not measured), providing some support for the conclusion that dietary protein quality is important. Yet more recently, Grandison et al. (2009) showed that adding a mixture of essential amino acids to a diet containing P:C 1:1.6 decreased lifespan in *Drosophila* to levels seen in files fed a P:C 1:1 diet, while enhancing fecundity. Adding methionine alone boosted fecundity but did not shorten lifespan, indicating that the lifespan shortening effects of a high dietary P:C relate to amino acid imbalance rather than some other property of yeast. The third study using the GF, that of Maklakov et al. (2008) on crickets, provides conclusive evidence that protein is a primary determinant of lifespan in that insect.

Maklakov et al. (2008) confined male and female field crickets, *Teleogryllus commodus*, to one of 24 chemically defined diets and measured intake, lifespan, female lifetime egg production, daily egg production, male lifetime calling effort (they sing to attract mates) and calling effort per night. Results for female lifespan and lifetime egg production are shown in 7.1. As with flies, both sexes of crickets lived longest on low P:C ratio diets, and died progressively earlier as P:C ratio increased. Males but not females demonstrated a reduction in lifespan at high intakes of low P:C diets; a result which was consistent with their greater propensity to lay down excess body fat on such diets. Similar costs of “obesity” on low P:C diets have been reported for other insects (Simpson et al. 2004; Skorupa et al. 2008). Fat deposition in response to excess ingested carbohydrate has been shown to be labile in laboratory selection experiments in insects; increasing in response to habitual shortage of carbohydrate across successive generations and decreasing in the face of persisting carbohydrate excess in the diet (Warbrick-Smith et al. 2006). Whereas longevity was greatest on low P:C diets in both sexes, female egg production was maximal at a much higher P:C ratio than sustained maximal calling in male crickets. When offered one of four complementary pairings of diets, males and females regulated nutrient intake to a trajectory that tended towards but failed to reach their respective nutritional optima for reproductive effort. The suggestion was made that this mismatch reflects unresolved intralocular sexual conflict in the control of nutrient intake, in which the regulatory responses of the two sexes are constrained by the common biology of nutrient regulation.

7.5 From Insects to Mammals

Insects are not mammals. The balance of opinion is that CR, not specific nutrient effects, is responsible for lifespan extension in mammals (Weindruch and Walford 1988; Masoro 2006). Therefore, is a geometric analysis of longevity needed in a mammalian model system? Two lines of evidence indicate that such an analysis is required. First, as discussed in detail by Simpson and Raubenheimer (2007), it is not possible to estimate response surfaces in nutrient intake space without providing a larger number of diet treatments than have been employed to date in experiments on the main model system in mammalian studies, rodents. Second, there are reports over many years that protein restriction, and of the amino acid methionine in particular, extends lifespan in rodents (Ross 1961; Orentreich et al. 1993; Miller et al. 2005; Zimmerman et al. 2003; Ayala et al. 2007).

A recent study has employed the GF on mice for the first time, although not in the context of aging and lifespan (Sørensen et al. 2008). Mice were either fed one of five isocaloric diets varying in the ratio of protein to carbohydrate, or one of four complementary food pairings, and food intake, growth and body composition were recorded over 32 days. Mice in choice experiments showed strong evidence of having separate regulatory systems for protein and carbohydrate, converging to a target P:C intake trajectory. In no-choice experiments they maintained intake of protein more constant than that of carbohydrate, with consequent elevated intake and increased body fat on low P:C diets. A full GF design would need to include variation in dietary lipid as well as protein and carbohydrate, extending the system to three dimensions. An efficient initial design would include around 30 treatments (10 P:C:L ratios and 3 total concentrations), which would need to be fed to mice throughout their lives. This is challenging but by no means intractable.

7.6 Mechanisms Linking Nutrition to Longevity

Specific nutritional effects clearly have an impact on longevity and perhaps too on the process of aging. The balance between protein and non-protein energy is emerging as a significant dietary factor in various ways. First, the fact that protein intake tends to be more strongly regulated than that of carbohydrate and fat [the protein leverage effect (Simpson and Raubenheimer 2005; Sørensen et al. 2008)] drives overconsumption of energy on low protein diets, promoting obesity and metabolic disorders with consequent effects on longevity. Second, when protein is eaten in higher then optimal quantities relative to carbohydrate it shortens lifespan; in insects certainly and perhaps too in rodents. The mechanisms involved are not known as yet, but several possibilities are suggested, including enhanced production of mitochondrial radical oxygen species (Sanz et al. 2004; Ayala et al. 2007), DNA and protein oxidative modification, changes in membrane fatty acid composition and mitochondrial metabolism (Ayala et al. 2007), changes in the relationship between insulin/IGF and amino acid signaling (e.g. TOR) pathways (Kapahi and Zid 2004; Kapahi et al. 2004; Powers et al. 2009), and toxic effects of nitrogenous breakdown products. Finally, an emerging body of work indicates that the balance between protein and non-protein energy in the diet determines longevity in the face of pathogen challenge through interactions with components of the immune system (Lee et al. 2006; Povey et al. 2009). How these various elements are interrelated will begin to emerge from GF analyses in which multiple response variables are mapped onto nutrient intake surfaces. We have recently proposed that the nexus of control for lifespan, obesity and associated metabolic disorders, and response to environmental stressors may lie in the interaction between the TOR and AMPK signalling pathways, with these serving as nutrient balance detectors (Simpson and Raubenheimer 2009).

7.7 Conclusion

The specific nutritional bases of dietary restriction remain incompletely resolved, but state-space models of nutrition (the GF) allow a better integrated understanding of the effects of diet on longevity and aging, the relationship between longevity and reproduction, and the metabolic, molecular and genetic mechanisms involved. Studies to date on insects give grounds for optimism for a geometric analysis of aging in mammals. Such a study will help resolve the extent to which the effects of diet restriction are mediated by caloric restriction or specific nutrient effects.

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Part II
Biochemical and Metabolic Mechanisms of Calorie Restriction

Chapter 8
Oxidative Stress, Dietary Restriction and Aging

Brian J. Merry and Catherine E. Ash

8.1 Introduction

While it is established that dietary restriction (DR) feeding regimes will extend the life span in a wide range of invertebrate and vertebrate species, and even slow the rate of aging in some, a full biochemical explanation of how this is achieved remains elusive (Merry 2002). A number of hypotheses have been proposed to explain the effect of DR on life span survival, particularly in short-lived rodent species, but the role of oxidative stress has attracted the greatest research attention (Barja 2004; Masoro 2005; Sanz et al. 2006). Although a significant body of data supports the conclusion that DR feeding regimes reduce the intensity of oxidative stress and damage in a comparison of tissues from older animals (Merry 2000), there is still controversy and inconsistency in the data that prevents its complete acceptance as a full explanation of the life span extension observed.

8.2 Dietary Restriction and Oxidative Damage

Many studies report that DR feeding regimes reduce the concentration of tissue oxidative damage biomarkers such as peroxidation of membrane lipids, protein carbonyl formation, oxidative damage to DNA bases and strand breaks in nDNA and mtDNA (Yu 1996; Merry 2000; Hamilton et al. 2001). There is frequently little detail published about the age-related profile that leads to this conclusion which is usually based on comparisons between older animals, neither is there significant detail provided about the functional consequences that result from these biochemical markers of oxidative damage. Very little is known of the kinetics regarding turnover and removal of oxidised proteins and lipids in older tissues but the conviction has developed that DR feeding induces a slower rate of accrual of tissue oxidative damage and that this is central to the extended survival observed.

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8.2.1 Are Dietary Induced Changes in Tissue Oxidative Damage Concentrations Reversible?

If the idea outlined above is correct then several questions arise. What effect on the life-time survival profile may be predicted when short-lived rodents are switched from control to DR feeding regimes and vice versa at different points in the life span? If the tissue concentration of oxidative damage is considered an irreversible biomarker of the process of aging, then switching feeding regimes should reveal a memory of the first feeding regime that is reflected both biochemically in the tissues, and in the resultant survival profile. If it does not, then this would be indicative that the differing intensities of tissue oxidative damage observed between control and DR animals may be a dynamic marker only of the specific rate of aging applying at the time of analysis, rather than as an indicator of the accrued life-time effects of the aging process.

Early studies indicated that just such a memory effect of limited periods of DR feeding could be identified in the survival profile when rats were switched from a short period of DR to control feeding (Yu et al. 1985; Cheney et al. 1983). Other studies could not verify such a memory effect of short periods of DR feeding, at least when these were implemented during the first year of life (Merry 1987). Conversely, an analysis of 36 survival studies in rodents using an intensity of DR feeding of between 40 and 50% of control food intake did show a highly significant positive covariance ($P<0.001$) between the duration of DR feeding and maximum survival. This was irrespective of whether DR feeding was the initial or secondary feeding regime (Merry 2002). When this dataset however is re-analysed after removing all data where the duration of DR feeding exceeded 1,000 days, the significant covariance observed is lost and there is no relationship between the duration of DR feeding and the maximum, or average last decile survival observed.

Recent studies designed specially to resolve this issue have concluded that there is little or no memory effect on the life-time survival profile in both *Drosophila* and the rat of the initial feeding regime when switching between DR and ad libitum feeding (Mair et al. 2003; Merry et al. 2008). Thus 1 year of full feeding prior to DR in rats has little downstream consequence for life-time survival. This contradicts the prediction that might be made if the concentration of oxidative damage in tissues was accruing at a faster rate under control, compared with DR feeding conditions, and that this damage was also irreversible. Switching feeding regimes from control to DR feeding at 1 year of age showed no intermediate position for the resultant survival profile and such animals exhibited a survival profile and life span extension indistinguishable from animals that had been on continuous DR feeding from 2 months of age (Merry et al. 2008). If therefore, oxidative stress and damage levels are important in determining the rate of aging, then to reconcile this proposal with the survival data requires that age-related tissue concentrations of oxidative damage are to a degree labile, and can be reversed by DR feeding. It appears that DR feeding regimes act to retard the rate of accumulation of a proportion of labile or reversible oxidative damage, but some tissue oxidative damage does not appear reversible and is not susceptible to dietary manipulation.

Oxidative damage as reflected by protein carbonyl concentrations in the mouse brain has been shown to respond to DR feeding in exactly this way. In the cortex, hippocampus, striatum, and midbrain of control mice, a significant age-related increase in protein carbonyl concentration was seen between 8 and 27 months of age. In comparison, 15-month old mice that have been maintained on 40% DR from 4 months of age exhibited significantly lower concentrations of protein carbonyls. The greatest effect was observed in the striatum where concentrations were 48% those of age-matched control brains. When mice of 15 months were switched between control and DR feeding regimes, the steady-state concentration of protein carbonyls reverted within 6 weeks to that characteristic of the new feeding regime (Dubey et al. 1996). It was concluded therefore, that the beneficial effects of DR upon life span may depend upon its ability to acutely reset the steady-state levels of oxidative stress and tissue damage. This dynamic response would explain the lack of a memory effect observed in the survival curves following cross-over studies between feeding regimes (Merry et al. 2008).

This interpretation has been confirmed by crossover studies conducted in C57BL/6 mice aged between 15 and 22 months where after 4 months of age, mice were maintained on either ad libitum or a 60% restricted feeding regime (Forster et al. 2000). When the feeding regimes were switched for a period of 6 weeks prior to tissue analysis, the chronic effect of DR to depress tissue carbonyl content in whole brain and the sulfhydryl content of the heart were found to be fully reversible within three to 6 weeks of initiating control feeding. The effect of chronic DR on the sulfhydryl content of the brain cortex was only partially reversible. When the converse switch from control to DR was made, a significant reduction in whole brain protein carbonyl content and cortex sulfhydryl concentration was seen following 6 weeks of DR feeding. The introduction of DR feeding did not affect the sulfhydryl content of the heart. This study confirms the chronic effect of DR feeding to reduce the age-accumulation of tissue oxidative damage but it also shows that some, but not all, of tissue oxidative damage responds dynamically to the feeding regime. It also demonstrates that the situation is complex and tissue oxidative damage is variable between tissues and the chemical nature of oxidative damage considered. Therefore the proposal that DR feeding regimes increase longevity by suppressing the lifetime accumulation of tissue oxidative stress and damage appears far too simplistic.

8.2.2 Does the Intensity of Oxidative Damage Correlate with Longevity?

Given the indication that some oxidative damage is a dynamic, rather than a permanent biomarker of aging, further questions can be considered. Does the intensity of DR feeding that is recognised to correlate with life span extension correlate with tissue oxidative damage concentration?

In a study of two DR feeding regimes of different intensity initiated at 17 months of age, muscle (*vastus lateralis*) from rats on 50% DR feeding showed better preserved histology as reflected by fibre number and fibre type when examined at 30–32 months age in comparison with muscle from rats on only 35% DR. These muscles also had fewer fibres staining negative for cytochrome oxidase or over-expressing succinate dehydrogenase, and fewer mtDNA deletions (Aspnes et al. 1997). There is therefore data to support a dose-response effect that the greater the intensity of DR, the more intense the effect is on preserving tissue function and ameliorating oxidative damage. These observations support conclusions drawn from survival data that up to the limit that a species can tolerate, the more severe the restricted feeding regime, the greater the effect on life span extension (Merry 2002). But again this is correlative data and it does not demonstrate that oxidative stress and damage levels determine life-time survival and the rate of aging.

8.2.3 How Late in the Lifespan is the Dynamic Effect of DR to Reverse Tissue Oxidative Damage Retained?

It is not known how late in life the dynamic response to DR feeding regimes persists both for the modification of the survival profile, and the reduction of oxidative damage. Three and a half months of DR feeding in 26.5 month old rats was sufficient to up-regulate the activity of the proteasome in rat liver (Goto et al. 2007) and DR feeding started at 19 months of age in male B6C3F1 mice still extended life span, and reduced tumour incidence within 2 months of initiation (Spindler 2005). Here, 75% of tissue gene expression changes induced by long-term DR were seen within 8 weeks of beginning DR feeding and, conversely, were lost within the same time-frame when DR fed animals were returned to control feeding (Spindler 2005). This cross-over study however, was initiated before the age where a progressive acceleration in the age-specific mortality rate is seen. Switching dietary regimes after the age when there is acceleration in the age-specific mortality rate of control animals has not been studied in any detail with respect to the effect on subsequent survival and tissue oxidative damage.

8.2.4 The Effect of Manipulating Oxidative Stress and Damage In Vivo on Longevity

A convincing demonstration in mammals that the reduced accrual of oxidative damage induced by DR feeding regimes is sufficient to extend survival and retard the development of the aged phenotype has still to be made. One approach to evaluate the role of oxidative stress as an important mechanism for determining the rate of aging has been through the use of gene knockout studies in mice. The manipulation of the exposure of the mitochondria to oxidative stress may be predicted to have significant downstream consequences on survival and markers of tissue oxidative damage. Mice heterozygous for the Sod2 gene (Sod2$^{+/-}$ mice) have been used to establish the age-related phenotype and life span survival where the mitochondrial

MnSOD activity was reduced by ~50% in all tissues throughout life (Van Remmen et al. 2003). The Sod2$^{+/-}$ mice had increased oxidative damage as evident from significantly elevated concentrations of 8oxodG in nuclear DNA (15% in heart and over 60% in liver when compared with the WT at 26 months of age) and also in mitochondrial DNA. This was associated with a 100% increase in tumour incidence (number of mice with tumours). Conversely, other biomarkers of oxidative stress and damage with age such as cataract formation, and glycoxidation end products such as carboxymethyl lysine and pentosidine in skin collagen, were not modified in their rate of change in the Sod2$^{+/-}$ mice. Importantly, mean and maximum survival was unaltered by the reduction in MnSOD activity and the increase in oxidative damage to DNA. No reports have been published as to whether DR feeding will extend longevity in Sod2$^{+/-}$ knockout mice.

In contrast, over-expressing the human form of catalase (50-fold) in the mitochondria of B6 mice resulted in an increase of 17% in median and maximum life span. This was associated with a significant reduction in cardiac pathology, in oxidative damage of skeletal and heart muscle DNA and in skeletal muscle mitochondrial DNA deletions (Schriner et al. 2005). Even though catalase was over-expressed 50-fold, the 17% increase in life span observed is significantly less than can be achieved by standard DR feeding regimes.

Mice over-expressing both human catalase in the peroxisome and Sod1 exhibited an 18.5% increase in medium life span and a 7% extension compared with littermates that only over-expressed the peroxisomal catalase. No effect on maximum life span was observed and heterozygous over-expression of CuZn Sod1 alone had no effect on survival (Huang et al. 2000). Thus the manipulation in vivo of the activity of key enzymes which dismutate and reduce mitochondrial superoxide and hydrogen peroxide, shows small and conflicting effects on life span survival, although a significant modification of tissue and mitochondrial oxidative damage can be demonstrated. Again the effect of DR feeding on longevity in these genetically manipulated mice is untested. Thus the evidence from such in vivo manipulations of tissue oxidative stress in rodents provides limited support only that oxidative stress and damage is important in controlling the rate of aging and is the mechanism through which DR feeding acts to increase longevity.

8.3 Dietary Restiction and Mitochondrial Function

There are many sites and enzymes within cells where free radicals are generated but mitochondria are considered to produce the majority of cellular reactive oxygen species (ROS) during normal metabolism (Beckman and Ames 1998). It is now established that in tissues from rodent species the major cause of reduced exposure to ROS under DR feeding regimes results not from an up-regulation of enzymatic and non-enzymatic antioxidants, but from a decrease in the rate of free radical generation (Sohal et al. 1994; Merry 2000; 2002; 2004; Sanz et al. 2006). To date, no genetic manipulation in rodents has succeeded in generating a strain in which reduced mitochondrial ROS generation is induced without other confounding metabolic effects, thereby allowing the effect on life span survival to be directly determined. A chronic reduction in mitochondrial ROS production has only been demonstrated in response to DR feeding in isolated mitochondria or cells using an in vitro approach. These observations are constrained therefore by the usual caveat that in vitro studies are frequently conducted under non-physiological conditions, specifically with respect to oxygen tension, substrate concentration, and the presence of inhibitors of complexes of the electron transport chain.

8.3.1 DR and Mitochondrial Reactive Oxygen Species Generation

A number of laboratories have confirmed in a range of tissues from DR animals that isolated mitochondria respire at the same rate as mitochondria isolated from age-matched control animals during either state IV (non-phosphorylating), or state III (phosphorylating) respiration. Therefore DR feeding regimes do not normally depress mitochondrial respiration rates, or whole animal standard metabolic rates, subsequent to a short period of adaptation (McCarter et al. 1985; McCarter and McGee 1988). Thus a lower rate of mitochondrial reactive oxygen species (ROS) generation is not simply the result of a reduction in the electron flux through the mitochondrial electron transport chain (ETC) in response to restricted energy intake.

In order to identify the mitochondrial adaptation to DR feeding that reduces superoxide generation, the approach of metabolic control analysis has been used. This is a systems analysis methodology that describes and quantifies the distribution of control within a complex homeostatic system. Top–down regulation analysis has been applied to quantify the response of mitochondria to the external effect of DR feeding and extraneous insulin supplementation. A full description of the theoretical background and practical methodology in the application of this approach has been published previously (Brand 1996; Lambert and Merry 2004).

This strategy recognises that it is not feasible to study all reactions and biochemical pathways simultaneously in a complex homeostatic system such as a mitochondrion, but by grouping related processes and intermediates into reaction blocks or modules, such a system can be simplified to make it amenable to real-time analysis. The study of Lambert and Merry (2004) suggested a mechanistic explanation for the lowered ROS generation observed in mitochondria isolated from tissues of DR animals. It had been recognized previously that in isolated heart and brain mitochondria, ROS generation during state IV respiration was dependent upon the degree of reduction of the ETC complexes as shown by the magnitude of the membrane potential (Δp) (Liu 1997; Korshunov et al. 1997; Hansford et al. 1997; Votyakova and Reynolds 2001). The proton gradient across the inner mitochondrial membrane is referred to as the protonmotive force (PMF) and is comprised of an electrical potential (voltage) and a pH component. Normally in studies of this type, the pH component of the protonmotive force is converted to a voltage and expressed with the electrical potential as a membrane potential (Δp). One explanation for the relationship of ROS generation to Δp is that under conditions of high Δp, intermediates of the ETC that transfer single electrons to oxygen become longer lived as a result of the reduced state of the ETC complexes. Thus the generation rate of ROS is considered to be extremely sensitive to the magnitude of Δp and a 10% reduction in the membrane potential can result in a 50% reduction in the rate of generation of ROS.

Unexpectedly DR feeding was observed to increase the proton leak rate of the inner liver mitochondrial membrane and in addition, to reduce the state IV membrane potential by 10–13%, sufficient to reduce the ROS generation rate by approximately 50% (Lambert and Merry 2004). It was concluded that a small, but significant decrease in the mitochondrial state IV membrane potential would explain the decreased rate of ROS observed. Intriguingly, this effect of DR feeding on the proton leak rate, the state IV potential and ROS generation, was reversible by exogenous insulin treatment over 1–2 weeks (Lambert and Merry 2004; Lambert et al. 2004). This ability of insulin to reverse the effect of DR feeding to reduce ROS generation and oxidative damage has been confirmed for heart, but not for liver mitochondria isolated from Wistar rats (Sanz et al. 2005).

This proposed functional explanation for the lowered ROS generated from mitochondria isolated from the tissues of DR animals has been somewhat controversial. In a series of studies of mitochondria isolated from rat ($FBNF_1$) liver and skeletal muscle, Bevilacqua et al. found either no effect on the proton conductance or a decreased leak in response to DR feeding (Bevilacqua et al. 2004; 2005). They did confirm that DR feeding induced a significant reduction in H_2O_2 generation which they explained resulted from a lowered respiration rate, or electron flux in mitochondria under DR feeding conditions. This observation contradicted a much earlier report from the same group where a detailed evaluation of the effect of DR feeding on state III and IV mitochondrial respiration rates using either a complex I and II substrate, revealed no depression in response to DR feeding (Weindruch et al. 1980). Further, they observed a chronic depression in the standard metabolic rate under DR feeding regimes in contrast to many other studies (McCarter et al. 1985; McCarter and Palmer 1992; Selman et al. 2005). In addition, they also reported an increase in the inner mitochondrial membrane concentration of uncoupling protein-3 (UCP3). An increase in UCP3 should normally be accompanied by an increase in mitochondrial proton leak if the UCP3 is activated to act as a protonophore. It is difficult therefore to reconcile both the increase in concentration of UCP3, and the higher membrane potential reported in response to DR feeding, with a 50% reduction in H_2O_2 generation given the recognised relationship between membrane potential and superoxide generation. In an attempt to reconcile these data it was argued that UCP3 does not function as a protonophore under DR feeding conditions, but instead has a role in fatty acid metabolism (Bevilacqua et al. 2005).

Given this controversy regarding the effect of DR on the mitochondrial inner membrane proton leak as an explanation for the lowered ROS generation observed, the study of Speakman and colleagues is of interest (Speakman et al. 2004). They reported a positive association between metabolic rate and lifespan in fully-fed mice. Mitochondria isolated from the skeletal muscle of mice in the upper quartile of survival had a higher proton conductance than mitochondria from mice from the lowest quartile. This association between increased proton leak and longevity is in agreement with the observation reported for DR fed animals (Lambert and Merry 2004). Fully fed individuals with high metabolism had mitochondria that were more uncoupled and as a result, had the greatest longevity.

In view of the lack of consistency in the published observations on mitochondrial proton conductance under DR feeding, and on the role of insulin, these studies have been repeated in the authors' laboratory for mitochondria isolated from a range of tissues. In every tissue evaluated, i.e. liver, hind limb skeletal muscle, heart, kidney and brown adipose tissue, a consistent effect of DR feeding to increase the proton leak, reduce the state IV mitochondrial membrane potential and reduced ROS generation was observed (Fig. 8.1). Further, in all experiments, even for insulin insensitive tissues, elevating plasma insulin concentrations by implanting mini-osmotic pumps in DR animals for 1 week prior to mitochondrial isolation, was sufficient to reverse these diet induced changes in mitochondrial function (Ash 2008) (Fig. 8.2). Therefore data from current research has confirmed and extended our previous observations regarding the functional adaptation of mitochondria in DR fed animals, the effect of which is to lower rates of ROS generation. Further, these studies have identified the molecular mechanism underlying this functional response.

8.3.2 Molecular Basis of DR-Induced Increased Proton Leak

Enhanced proton conductance of the inner mitochondrial membrane of mitochondria is normally considered to result from activation of uncoupling proteins, (UCP-2 or UCP-3), or through structural changes in membrane lipids particularly the degree of un-saturation that affects membrane order, or through the activation of membrane protein translocases. Our present studies show that the increased membrane proton conductance results from increased protonophoric activity during state IV respiration of the adenine nucleotide translocase (ANT) (Ash 2008). This activation is associated with reduced rates of H_2O_2 generation and the lowered membrane potential reported previously. During state III respiration, when ADP is being phosphorylated to ATP, the ANT exchanges ADP and ATP across the inner mitochondrial membrane. During state IV respiration, when phosphorylation of ADP is not occurring, it acts as a variable proton leak channel across the inner mitochondrial membrane linking the inter-membrane space and the mitochondrial matrix.

Specific inhibition of the ANT in mitochondria of DR animals consistently increased H_2O_2 generation to control rates under state IV respiration conditions (Ash 2008). Rates of H_2O_2 generation are reduced by DR feeding not only when supported by a complex II, but also a complex I substrate. The reduction of H_2O_2 generation when a complex I substrate is used to support respiration is particularly important as reverse electron flow, which occurs when respiration is supported by

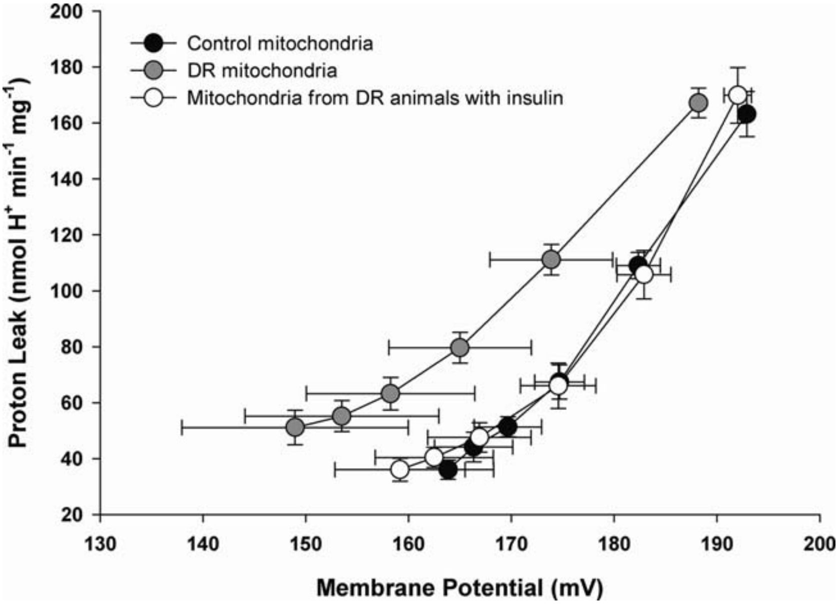

Fig. 8.1 The effect of DR feeding on the mitochondrial proton conductance of the inner mitochondrial membrane at varying driving membrane potentials is shown for liver mitochondria isolated from animals of 8–9 months of age. DR feeding at 55% the ad libitum intake was started at 1.5 months of age. The response to the restricted feeding regime is to move the non-ohmic (non-linear) leak curve to the left at any given driving potential, indicative of a greater proton leak rate across the membrane. Importantly for ROS generation, the highest membrane potential achieved during state IV respiration is significantly lower in mitochondria from DR animals than is seen in mitochondria isolated from the liver of control animals. A third leak profile is shown where DR age-matched animals have received exogenous insulin prior to mitochondrial isolation over a 1 week period delivered from a subcutaneously implanted Alzet mini-osmotic pump (27 μmol/L insulin at a pump rate of 0.5 μL/h). A significant elevation of plasma insulin concentration ($P < 0.02$) from 0.30±0.04 to 0.48±0.06 μg/L is observed (Lambert and Merry 2004). This plasma insulin concentration is still significantly lower than recorded in control animals 1.19±0.13 μg/L. It is not possible during the first year of life to elevate plasma insulin concentrations in DR rats above this concentration without the risk of inducing hypoglycaemia. An associated elevation in plasma glucagon concentrations is observed in DR animals, which is reversed by elevating plasma insulin. Elevation of plasma insulin concentrations in DR fed animals reverses the increase in proton leak and lowered state IV membrane potential induced by DR feeding to the profile observed for control animals

a complex II substrate and when rotenone is not included in the reaction buffer, may not be physiologically relevant. Only in mitochondria isolated from brown fat was the increase in membrane proton conductance induced by DR feeding dependent upon the activation of an uncoupling protein, UCP-1 (Ash 2008). This is an observation that would be predicted from the moderate lowering of the core body temperature of rodents maintained on DR feeding regimes (Cheney et al. 1983; Holehan and Merry 1986; Lambert et al. 2004).

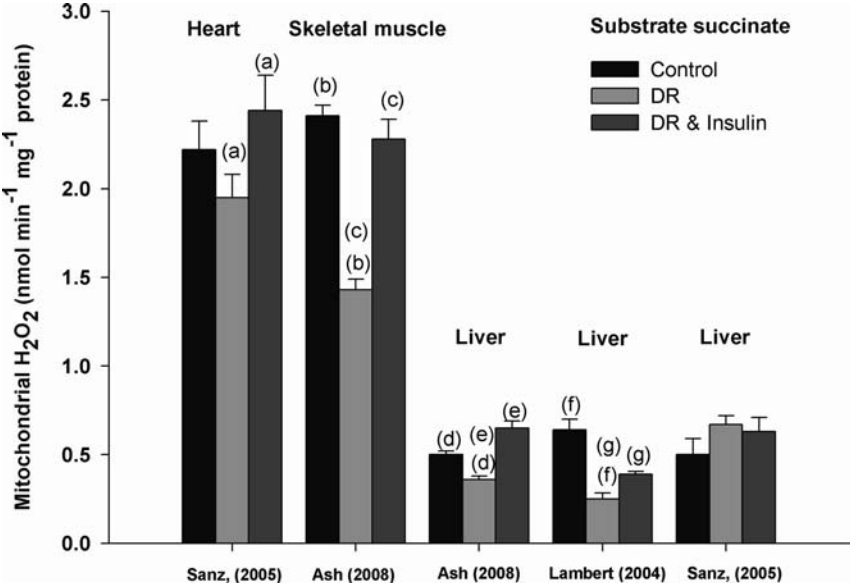

Fig. 8.2 The effect of DR feeding and elevating plasma insulin concentrations in DR rats on H_2O_2 generation rates for mitochondria isolated from heart, hind limb skeletal muscle and liver is shown for three separate studies, i.e. (Lambert and Merry 2004; Sanz et al. 2005; Ash 2008). In the study of Sanz et al. (2005), Wistar male rats of 10 weeks of age were restricted by 40% from control feeding for 6 weeks before mitochondrial isolation whereas for DR rats supplemented with insulin, daily s.c. injections of fast and slow release insulin were given daily during the last 2 weeks before mitochondrial isolation. In the studies of Lambert and Merry (2004) and Ash (2008), 6–8 month old male BN rats were used where restricted feeding (55% control intake) was started at 1.5 months of age. Exogenous insulin was supplied by implanted mini-osmotic pumps (s.c.) for 1 or 2 weeks prior to mitochondrial isolation. DR feeding in the studies of Lambert and Merry (2004) and Ash (2008) lowered plasma insulin concentrations by 75% whereas in the study of Sanz et al. (2005), the much shorter period of DR reduced plasma insulin by only 20%, and it was possible in this study to elevate plasma insulin back to control concentrations without symptoms of hypoglycaemia (see also legend to Fig. 8.1). In all studies and tissues with the exception of liver in the Sanz et al. (2005) short-term feeding protocol, DR feeding reduced significantly the generation of H_2O_2 when mitochondrial respiration was supported by succinate and this effect was fully or partially reversed by elevation of plasma insulin concentrations. Group means ± s.e.m are shown and columns with the same letters are significantly different for group means at the following probabilities, (a,f,g, $P<0.05$; b,c,d,e, $P<0.001$). Animal numbers were 7–10 (Sanz et al. 2005), 14, 9 & 3 for control, DR and DR & insulin (Lambert and Merry 2004) and 6 for all groups (Ash 2008)

8.4 General Discussion

Although described only briefly above, it is now possible to construct a detailed mechanism that explains the lower ROS generation observed in isolated mitochondria from tissues of DR rodents, the consequence of which is considered to be the reduction in tissue oxidative stress and damage observed in vivo. The relevance of these observations however, or indeed oxidative stress and damage to the determination of longevity and the rate of aging, remains uncertain. The downstream target of cellular and mitochondrial ROS that may modify the rate of aging remains to be clarified. There is inconsistency in the data relating oxidative modification of DNA bases to longevity, and only correlative data relating oxidative induced deletions in mtDNA to normal aging. Alternative prospective targets for cellular ROS that may contribute to controlling longevity are apoptosis and cell loss, stem cell senescence and differentiation, epigenetic modification, activation of redox sensitive transcription factors, telomeric deletions and the loss of proteosome efficiency. The role of ROS therefore in determining the rate of aging remains contentious, not least because of the high oxidative tissue damage levels reported for the naked mole rat, the longest lived rodent with a maximum lifespan of greater than 28.3 years (Andziak et al. 2006).

Attempts to resolve this debate concerning the importance of oxidative stress in determining the rate of aging and whether its amelioration underlies the increased longevity observed with DR feeding have frequently relied on studies where the diet has been supplemented with an antioxidant. Supplementation of the diet with a range of small molecular weight antioxidants has met with little or no success in mimicking the effect of DR on survival. This conclusion has been confirmed recently for the putative powerful antioxidant α-lipoic acid (Lee et al. 2004; Merry et al. 2008). Interestingly, ad libitum feeding a diet supplemented with α-lipoic acid can induce the extended survival profile characteristic of DR feeding provided it follows a period of normal DR feeding (optimum period in the rat is approximately 10 months) before being added to the diet. Lipoic acid appears to block the normal dynamic response of rodent survival to changes in feeding regime where the extended survival induced by DR is quickly lost on return to full feeding with the non-supplemented diet (Table 8.1).

This ability to fix the original survival trajectory induced by the initial feeding regime is also seen in animals fed ad libitum with the α-lipoic acid supplemented diet, prior to the switch to DR feeding with a non-supplemented diet. Here the normal transition to the extended survival trajectory induced by DR feeding is prevented, even though the diet is no longer supplemented with lipoic acid after the transition to the DR feeding regime (Table 8.1). Thus lipoic acid can exert a persistent effect to modify survival for over a year after ceasing to supplement the diet (Merry et al. 2008). The mechanism underling this persistent effect of lipoic acid on survival is unknown but may reflect epigenetic modification of tissue gene expression profiles. Lipoic acid is a recognised inhibitor of bulk histone deacetylation with the potential to modify gene silencing and expression. Based however, on the kinetics of its rapid clearance from plasma and tissues, it appears unlikely to be acting directly through its putative role as an antioxidant to protect cells from oxidative stress. Lipoic acid is recognised to bind to the antioxidant response element (ARE) and to up-regulate γ-glutamylcyteine ligase the rate-limiting enzyme of GSH synthesis and of Phase II Detoxification. ARE is the cis-acting enhancer sequence that transcriptionally regulates Phase II detoxification that controls more than 200 antioxidant and detoxification enzymes through the Keap1–Nrf2

Table 8.1 Dietary groups with the associated median survival and 95% confidence limits, mean survival ± sem and the hazard ratio

Dietary group description	Group size	Median survival (95% CI) (days)	Mean survival (std error) (days)	Hazard ratio[a]	*P*
Control animals fed ad libitum the CRM diet throughout life	102	926 (909–943)	854 (22)	1.000	NA
Fed a restricted intake of the CRM diet from 2-months to maintain body weight at 55% age-matched control animals	75	1,047 (930–1,163)	1,025 (25)	0.259	<0.0001
Animals fed ad libitum the CRM diet supplemented with R/S racemic mixture of α-lipoic acid from 2-months of age	75	900 (839–961)	858 (27)	0.983	NS
DR fed the CRM diet until 12-months, then DR fed the α-lipoic acid supplemented diet	24	1,125 (1,078–1,172)	1,068 (38)	0.178	<0.0001
Ad libitum fed CRM diet, animals switched to DR feeding at 12-months	25	1,031 (1,007–1,055)	1,000 (33)	0.235	<0.0017
DR fed the CRM diet from 2- to 12-months, then switched to ad libitum feeding	25	975 (935–1,015)	914 (44)	0.620	NS
Animals fed ad libitum α-lipoic acid supplemented diet 2- to 12-months, then switched to DR feeding the CRM diet (no α-lipoic acid supplementation after 12-months)	25	934 (874–994)	859 (57)	0.865	NS

Table 8.1 (continued)

Dietary group description	Group size	Median survival (95% CI) (days)	Mean survival (std error) (days)	Hazard ratio[a]	P
DR fed the CRM diet from 2- until 12-months, then switched to ad libitum feeding the α-lipoic acid supplement CRM diet	25	1,041 (895–1,187)	1,009 (34)	0.353	<0.0044

[a] The Hazard Ratio is the decreased life-time risk of death of the dietary groups when compared with that of the control group that was fed ad libitum, the CRM non-supplemented diet. The hazard ratio was determined by the Peto log-rank analysis with the control group survival set to 1.0. Values lower than 1.0 represent a slower rate of death than observed in the control animals. Statistical comparisons show the effect of the dietary treatment on increasing survival in comparison with the control, fully fed group as identified by log-rank analysis with statistical significance set at $P \leq 0.05$ (adapted from Table 1 in Merry et al. 2008).

pathway (Merry et al. 2008). Thus alternative pathways exist whereby lipoic acid could modify survival through a persistent effect on the redox status of tissues. If these observations on survival are reproducible and can be extended to other model organisms, the use of lipoic acid supplementation after DR feeding offers an alternative model system to evaluate the role of oxidative stress and other mechanisms that may explain the effect of DR to extend survival and retard the rate of aging.

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Chapter 9
Calorie Restriction Mimetics and Aging

Brian J. Morris

9.1 Introduction

It is well established that caloric restriction (CR) extends lifespan of all species tested. But who would be willing to CR themselves? Instead, it would be far simpler to be able to take a pill that mimics the effects of CR and thereby make us healthier, prevent disease and allow us to live longer. But is this feasible? The present chapter discusses the latest evidence for such a possibility, albeit probably not as powerful as CR.

Within the plant kingdom one can find various "CR mimetics" (Howitz et al. 2003) – plant molecules that activate similar pathways as some of those stimulated by CR. As a result they confer health benefits and, just as in other species, could have the potential to prolong human lifespan, or at least help prevent premature death from common diseases of aging. These chemicals are often pigments and increase in concentration in various plants in response to natural stressful events such as climate change (cold, heat or drought), ozone, UV light, and fungal infection (where they serve as natural antibiotics). Animals that feed on the plants have evolved an ability to respond to the plant chemicals in advance and so survive the environmental change (Howitz and Sinclair 2008). This is known as xenohormesis (Howitz et al. 2003; Lamming et al. 2004). What is more, the plant chemicals are often safe (Corson and Crews 2007) – this, despite many of these having actions on multiple targets in cells.

Pharmacological applications for bioactive plant molecules are nothing new. Salicylates (whose acetylated derivative, aspirin, is used widely) arose when the antipyretic and anti-inflammatory effects of willow bark extracts were noted in 1763 (Howitz and Sinclair 2008). So could it be that we are on the brink of yet another era when a further class of natural compounds is set to be used just as commonly. In fact we already consume such natural chemicals in our diet, especially when it is

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a healthy one rich in vegetables and fruit, confirming that these are indeed likely to be beneficial.

There are numerous different natural bioactive chemicals. These have a vast array of different, and often overlapping, beneficial actions on the body. Most of the ones referred to in this chapter are polyphenols. These are a broad class of chemicals found in plants consumed by animals. Members of the flavonoid family of polyphenols are represented strongly (Fig. 9.1).

Flavonoids are a group of compounds containing a characteristic aromatic nucleus. The polyphenol content of a plant may thus represent a chemical signature of its environment (Howitz and Sinclair 2008). The ability of animals to respond to these stress-induced phytochemicals in beneficial ways has likely been an evolutionary one (Howitz and Sinclair 2008). The response in animals involves activation of enzymes and receptors in their own stress-response pathways. In the natural habitat, this allows the animals to get a head start in surviving adversity before conditions deteriorate further. For short-lived species the organisms might then survive to reproduce. Such responses are very different from those from low doses of toxins, such as are present in some plants and cause cellular damage. In fact most natural plant polyphenols have remarkably low toxicity, as shown when high

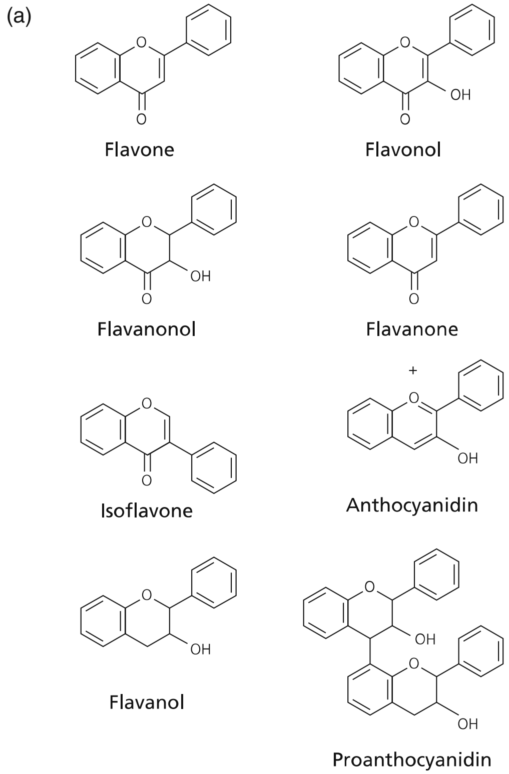


Fig. 9.1 Dietary polyphenols, showing the (**a**) classification and (**b**) ring structures of the various flavonoids (Modified from Magrone et al. (2008))

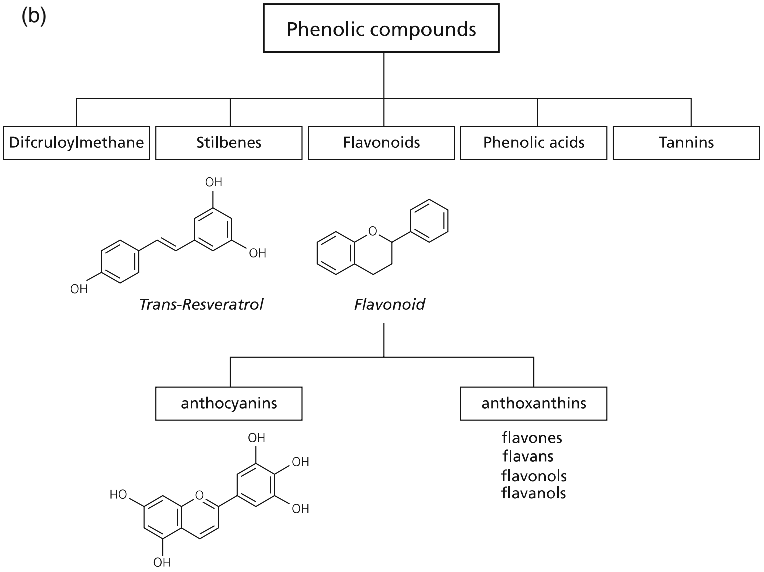


Fig. 9.1 (continued)

doses are administered (Baur and Sinclair 2006). The concentrations of polyphenols capable of extending lifespan in laboratory experiments (approx. 10 μM) are similar to the levels found in leaves and fruit of plants under stress.

The chemical classes of polyphenols synthesized by plants as secondary metabolites include flavones, isoflavones, stilbenes, catechnics, acanthocyanidians and chalcones (Fig. 9.1). In plants they serve to deter herbivores, filter ultraviolet light, are antioxidants, antibiotics and fungicides. They are also involved in signaling pathways within the cell. In animals it is often thought that their benefit occurs because they act as antioxidants. This is at best far too simplistic and in general is not supported by the evidence. Their ability to decrease damaging reactive oxygen species (ROS) is indirect, involving induction of defense enzymes such as heme oxygenase. Examples of polyphenols that have been studied extensively are resveratrol, catechins, genistein, and quercetin. The literature on each is enormous (Table 9.1), so not all of the findings can be covered here.

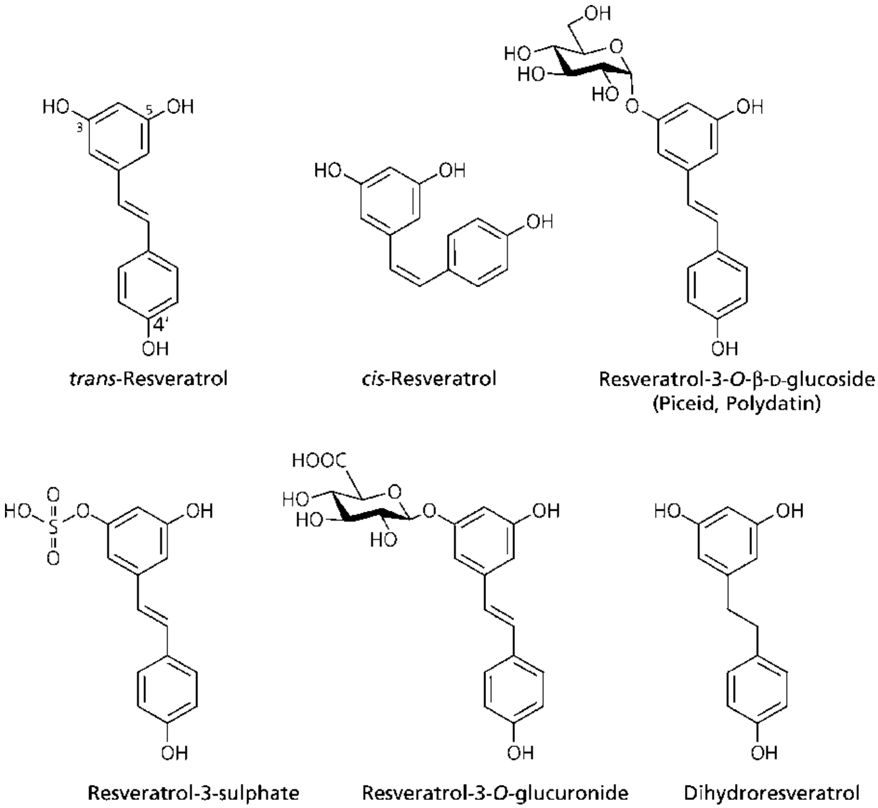
Table 9.1 Number of publications retrieved by a PubMed search*

Keyword	Number of publications
Resveratrol	2,672
Catechin	4,835
Genistein	7,696
Quercetin	6,450

*Conducted 1 April, 2009.

9.2 Resveratrol

The most famous polyphenolic flavonoid, because of its potency and the fact that it is present in red wine, is resveratrol (also known as either *trans*-3,5,4′-trihydroxystilbene or 5-[(E)-2-(4-hydroxyphenyl)-ethenyl]benzene-1,3-diol; $C_{14}H_{12}O_3$) (Fig. 9.2)



trans-Resveratrol *cis*-Resveratrol Resveratrol-3-*O*-β-D-glucoside (Piceid, Polydatin)

Resveratrol-3-sulphate Resveratrol-3-*O*-glucuronide Dihydroresveratrol

Fig. 9.2 Structure of resveratrol and related compounds in red wine (*upper row*) and metabolites (*lower row*) (Modified from Baur and Sinclair (2006))

It is found in berry fruit such as grapes (50–100 mg/g), where its concentration is highest in skins and seeds, blueberries, mulberries, red wine, peanuts, rhubarb and, in all, over 70 species of plant covering 32 genera (Harikumar and Aggarwal 2008). Resveratrol is the active ingredient in the traditional medicine Kojo-kon, made from the dried roots of Japanese knotweed. It is generally thought that resveratrol mediates its effects largely, although not exclusively (Zhang 2006; Alvira et al. 2007), through its ability to activate NAD^+-dependent class III deacetylases known as sirtuins, of which there are seven members (Sirtl through Sirt7) in humans and other mammalian species (Yamamoto et al. 2007). There is, nevertheless, some data to dispute involvement of sirtuins in the effects of resveratrol (reviewed in (Pirola and Fröjdö 2008)). One sirtuin-independent effect of resveratrol involves inhibition of phosphoinositide 3-kinase (PI3K) (Fröjdö et al. 2007), a key component of the insulin signalling pathway that when suppressed extends lifespan (reviewed in Morris (2005)).

Resveratrol also binds to, and in most cases inhibits, protein kinase C, aromatase, hetero-dimeric αVβ3 integrin, multidrug resistance protein-1, β-lactoglobulin, human DNA topisomerase II, plasma lipoprotein, DNA polymerase α and δ, myeloperoxidase, tubulin, oestrogen receptor α and β, F1-ATPase, sulfonyl urea receptors, cytosolic alcohol dehydrogenase, amyloid fibrils, dihydronicotinamide riboside quinone reductase 2, nucleic acids and human serum albumin (reviewed in Harikumar and Aggarwal (2008)). Some of the pathways and processes activated by both CR and resveratrol are shown in Fig. 9.3.

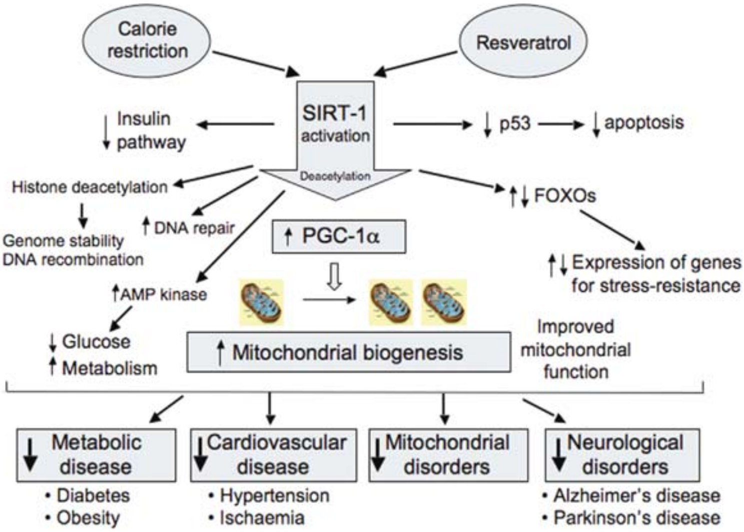


Fig. 9.3 General pathways and processes activated by calorie restriction and resveratrol

Resveratrol and other sirtuin activators bind to hydrophobic pockets in sirtuin proteins via van der Waals contacts and H-bonds involving hydroxyl groups (Milne et al. 2007; Howitz and Sinclair 2008). They do not compete with the nucleotide substrates of the enzymes, but bind elsewhere on the protein (Howitz and Sinclair 2008). Of various natural small molecule sirtuin activators tested, resveratrol is the most potent (Howitz et al. 2003). The concentration of polyphenols such as resveratrol are, moreover, elevated in cultivars of *Vitis vinifera* when growing conditions are harsher, consistent with the xenohormesis hypothesis (Howitz et al. 2003; Lamming et al. 2004; Morris 2007; Howitz and Sinclair 2008; Morris 2008).

Resveratrol modulates the function of quite a number of enzymes and receptors, as well as various transcription factors (Gross et al. 2008). It is nontoxic, and studies in vitro or in animal models of disease have ascribed a wide array of health benefits to this chemical (Baur and Sinclair 2006; Westphal et al. 2007). The variety of gene products modulated by resveratrol is depicted in Fig. 9.4 and many of these will now be discussed.

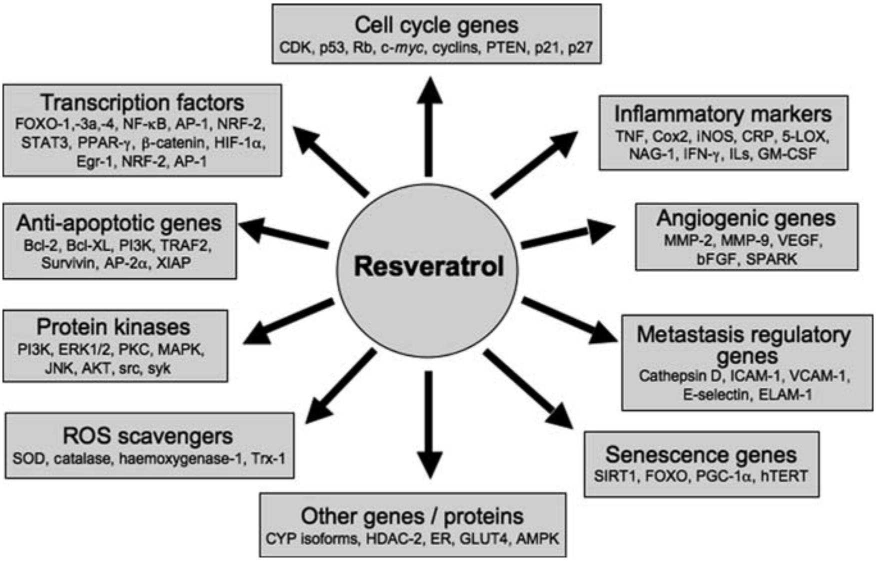

Cell cycle genes
CDK, p53, Rb, c-*myc*, cyclins, PTEN, p21, p27
Transcription factors
FOXO-1,-3a,-4, NF-κB, AP-1, NRF-2, STAT3, PPAR-γ, β-catenin, HIF-1α, Egr-1, NRF-2, AP-1
Inflammatory markers
TNF, Cox2, iNOS, CRP, 5-LOX, NAG-1, IFN-γ, ILs, GM-CSF
Anti-apoptotic genes
Bcl-2, Bcl-XL, PI3K, TRAF2, Survivin, AP-2α, XIAP
Resveratrol
Angiogenic genes
MMP-2, MMP-9, VEGF, bFGF, SPARK
Protein kinases
PI3K, ERK1/2, PKC, MAPK, JNK, AKT, src, syk
Metastasis regulatory genes
Cathepsin D, ICAM-1, VCAM-1, E-selectin, ELAM-1
ROS scavengers
SOD, catalase, haemoxygenase-1, Trx-1
Senescence genes
SIRT1, FOXO, PGC-1α, hTERT
Other genes / proteins
CYP isoforms, HDAC-2, ER, GLUT4, AMPK

Fig. 9.4 Categories of proteins, with examples, whose expression is altered by resveratrol (Modified from Harikumar and Aggarwal (2008))

9.2.1 Cancer

Resveratrol is a phytoestrogen, having structural similarity to diethylstilbestrol. Over a decade ago, when given topically to mice, resveratrol was found to reduce skin tumors by 98% (Jang et al. 1997). In many rodent models of various cancers initiation and growth of tumors has since been found to be prevented by its systemic administration (Baur and Sinclair 2006). This included colon cancer in rats using a dose of 200 μg/kg body weight (Tessitore et al. 2000), a dose of polyphenols obtainable from dietary sources (Baur and Sinclair 2006). As well, subcutaneous neuroblastomas, with 70% of mice surviving after administration 40 mg/kg (Chen et al. 2004).

Protective effects in prostate cancer cell lines and potential mechanisms involved have been studied extensively (reviewed in Harikumar and Aggarwal (2008)). In LNCaP cells over 1,600 transcripts exhibited an alteration in expression 6 h after treatment (Jones et al. 2005). Resveratrol was, moreover, able to prevent prostate cancer in a mouse model (Harper et al. 2007). The Sirt1 gene, *SIRT1*, is overexpressed in prostate cancer and its inhibition by nicotinamide, sirtinol or RNA-mediated interference inhibits the growth of prostate cancer cells, but not that of normal prostate cells (Jung-Hynes et al. 2009). It was suggested that Sirt1, by inhibiting the activation of forkhead box subgroup O (FOXO)-1 transcription factor, can contribute to the development of prostate cancer.

In breast cancer no effect was seen in one study (Bove et al. 2002), but beneficial effects were apparent in others (Whitsett et al. 2006; Su et al. 2007). Tumor growth and angiogenesis, an important component of cancer progression, was inhibited by resveratrol in nude mice with human breast cancer xenografts, and in breast cancer cells in vitro resveratrol increased apoptosis and lowered vascular endothelial growth factor (VEGF) (Garvin et al. 2006). Some breast cancers are caused by mutation in the gene *BRCA1*. Brca1, the product of this gene, binds to the *SIRT1* promoter leading to stimulation of Sirt1 production, so explaining why, in these breast cancers, Sirt1 is reduced (Wang et al. 2008b). Resveratrol treatment, by activating Sirt1 and inhibiting expression of Survivin, reduces growth of BRCA1 breast cancer cells in vitro and in vivo, so implicating resveratrol in therapy for BRCA1-related breast cancers.

Resveratrol also offers potential benefits to cancers of the liver, pancreas, stomach, intestines, colon and rectum, as well as in leukaemia, lymphoma and myeloma. The details of the effects of resveratrol on cells and molecules in these cancers has been reviewed (Harikumar and Aggarwal 2008).

The anti-cancer effects of resveratrol include suppression of cyclooxygenase activity and gene expression and suppression of ornithine decarboxylase expression, enzymes that are each needed for angiogenesis (Baur and Sinclair 2006), with 2.5–100 mg/kg being able to inhibit tumor-induced neovascularization vital for solid tumor growth (Kimura and Okuda 2001; Tseng et al. 2004).

Resveratrol also alters expression of enzymes involved in drug metabolism, such as cytochrome P450s (Baur and Sinclair 2006). By upregulating Phase II enzymes, resveratrol reduces environmental carcinogens in the body. This effect might, however, adversely affect the pharmacokinetics of drugs (Baur and Sinclair 2006). Quinone reductase II, a high affinity target for resveratrol (Buryanovskyy et al. 2004), by increasing electrophile concentrations, could induce expression of Phase II enzymes (Baur and Sinclair 2006).

Because of its anti-proliferative and pro-apoptotic effects (Aggarwal et al. 2004), resveratrol is able to downregulate cell cycle proteins and increase apoptosis of tumor cells (Baur and Sinclair 2006). Cell cycle proteins modulated include retinoblastoma protein (Rb), cyclin-dependent kinases (CDKs), cyclins and c-myc (reviewed in (Harikumar and Aggarwal 2008)). It induces apoptosis of tumor cells by increasing p53 and suppressing Bcl-2, Bcl-X_L, XIAP, Survivin and TRAF2 (reviewed in Harikumar and Aggarwal (2008)).

Resveratrol inhibits angiogenesis and metastasis and this involves effects on matrix metalloproteins (MMPs), VEGF, cathepsin D, ICAM-1 and E-selectin. Cycling cells are more susceptibile to resveratrol (Ferry-Dumazet et al. 2002), so it might sensitize tumor cells to apoptotic factors such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Fulda and Debatin 2004).

The anticancer effects of resveratrol might also involve its antioxidant effects, where ROS, by damaging DNA and other macromolecules, contribute to the initiation and progression of cancer (Baur and Sinclair 2006). Such effects could be direct or could be a consequence of the upregulation by resveratrol of antioxidant enzymes – superoxide dismutase (SOD), catalase, glutathione peroxidase and haemoxygenase (reviewed in Harikumar and Aggarwal (2008)).

Inflammation is a feature of cancer, and resveratrol is able to downregulate inflammatory biomarkers such as tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (Cox-2) inducible nitric oxide synthase (iNOS), C-reactive protein (CRP), interferon-α and several interleukins (reviewed in Harikumar and Aggarwal (2008)).

Phase I clinical trials of resveratrol are in progress in the USA and UK with up to 7.5 g/day (Baur and Sinclair 2006). The results of these should indicate its clinical efficacy in cancer treatment (Baur and Sinclair 2006).

9.2.2 Cardiovascular Disease

It has been suggested that red wine and grape extracts might confer a protective effect against cardiovascular disease (Bohm et al. 2004). Benefits could include improvement of serum cholesterol profile and triglyceride concentrations, reduced platelet aggregation, anti-atherosclerotic effects, reduction in lipid peroxidation, decreased endothelin-1, protection of endothelial cells against apoptosis, relaxation of blood vessels, reduction in blood pressure, as well as oxidative stress and, in hypertensive animals, prevention of end-organ damage (reviewed in (Baur and Sinclair 2006; Perez-Vizcaino et al. 2006; Harikumar and Aggarwal 2008)). Could such effects offer a partial explanation for the "French Paradox"? – i.e., a lower rate of cardiovascular disease in the French arising from moderate consumption of red wine in the face of a diet traditionally high in animal fat (Renaud and de Lorgeril 1992).

Indeed, red wine and grape extracts contain polyphenols, which include monomeric flavanols, flavonols, phenolic acids, anthocyanins, proanthocyanins, and stilbene derivates such as resveratrol. Resveratrol (Orallo et al. 2002), together with the anthocyanididin delphnidin (Andriambeloson et al. 1998) and the flavanol quercetin (Flesch et al. 1998), mediate the beneficial physiological effects conferred by red wine polyphenol extracts (RWPE).

Resveratrol prevents platelet aggregation by inactivating selectively the prostaglandin H_2 synthase, Cox-1, as opposed to Cox-2 (Szewczuk et al. 2004). This action is similar to the way aspirin exerts its cardioprotective effects. As well, resveratrol is a potent inhibitor of the peroxidase reactions of Cox-1. Its inhibition of Cox-2, the isoform targeted by non-steroidal anti-inflammatory drugs, is weak, and is, moreover, confined to cyclooxygenase activity (Szewczuk et al. 2004). The inhibitory effect of resveratrol involves peroxide substrate and is accompanied by concomitant oxidation of resveratrol at the peroxidase active site. The usual effect of

Cox-1 is in thromboxane A_2 synthesis, the latter being a potent inducer of platelet aggregation and vasoconstriction (Mukherjee et al. 2001). Inhibition of Cox-2 on the other hand can increase thrombus formation (Mukherjee et al. 2001).

As well as inhibiting thromboxane A_2 formation to cause vasodilation, the vasodilator effect of resveratrol is also caused by stimulation of Ca^{2+}-activated K^+ channels (Li et al. 2000), as well as inhibition of vascular NADH/NADPH oxidase activity, so increasing nitric oxide (NO) signalling in the endothelium, with the result that superoxide production is reduced and NO inactivation is suppressed (Orallo et al. 2002). Unlike other flavonoids, resveratrol increases endothelial nitric oxide synthase (eNOS) and iNOS gene expression (Leikert et al. 2002; Wallerath et al. 2002; Das et al. 2005; Leighton et al. 2006). Neither resveratrol, nor other specific polyphenols affect L-citrulline production by vascular endothelial cells, whereas RWPE do (Leikert et al. 2002). The increase in eNOS in response to RWPE and resveratrol means an elevation in vasodilator NO generation in blood vessel walls (Räthel et al. 2007). Moderate consumption of red wine in mice improves ischaemia-induced neovascularization in high-cholesterol conditions by restoring the Akt (protein kinase B)–eNOS–NO pathway and by increasing the number and function of endothelial progenitor cells (Lefèvre et al. 2007).

Resveratrol inhibits Ca^{2+} channels and Ca^{2+} influx into platelets, inhibits adhesion of platelets to type I collagen, decreases collagen aggregation induced by platelets, inhibits type I collagen mRNA, collagen and thrombin formation, prevents ADP-induced mitogen-activated protein kinase (MAPK) activation, reduces adhesion of thrombin and ADP-activated platelets to fibrinogen, and promotes fibrinolysis, so protecting against atherosclerosis (Shen et al. 2007).

When RWPEs (160 μg/ml) from 180 red wines and a few whites were tested, marked differences in their ability to increase eNOS expression were observed (Räthel et al. 2007). The most potent effect was seen with a French Merlot (6.1-fold increase), followed by Syrah and French Pinot noirs (4-fold). White wine extracts had little effect. It is likely that climate, rather than the particular cultivar or the growing area is what determines RWPE potency (Morris 2007; Räthel et al. 2007). The 600 μg/ml of RWPE corresponded to 1–10 μg resveratrol per 750 mL of red wine, (1–10 μmol/L). Even though 10 μM resveratrol increases eNOS promoter, but not enzyme activity significantly, other constituents in RWPE make an important contribution (Räthel et al. 2007). It may be that the various constituents work in synergy (Wallerath et al. 2005). Polyphenol extracts of grape juice are less effective, but only slightly so, meaning that while vinification might increase polyphenol content, it is not a prerequisite for obtaining active extracts (Räthel et al. 2007). Seasonal and regional variation in climatic conditions might be behind the variation in potency of RWPE from different geographic locations and vintages. In accord with the xenohormesis hypothesis, a cold season, for example, might be responsible for higher polyphenol content of the wines in that particular year (Lamming et al. 2004).

Risk of heart disease and myocardial infarction is increased by oxidation of low density lipoprotein (LDL) particles. The latter effect is prevented by resveratrol, which chelates copper and scavenges ROS (Frankel et al. 1993). Resveratrol can, moreover, reduce infarct size (Ray et al. 1999). Other components of red wine are, however, more effective scavengers of ROS (Fremont et al. 1999). The fact that one can detect resveratrol in LDL particles after consumption of red wine (Urpi-Sarda et al. 2005) is consistent with its ability to prevent peroxidation of lipids and other macromolecules (Baur and Sinclair 2006). Resveratrol can, moreover, limit accumulation of cholesterol by human macrophages. It does so by activating transcription factors that target the nuclear liver receptor-α (LXR-α) gene, and genes influenced by LXR-α, namely cholesterol efflux genes *ABCA1* and *ABCG1*, and repressing expression of the lipid uptake genes lipoprotein lipase and scavenger receptor SR-AII (Sevov et al. 2006). In atherosclerosis MMPs are induced by upregulation of extracellular matrix metalloproteinase inducer (AMMPRIN) in monocytes/macrophages and coronary smooth muscle cells in response to proatherogenic stimuli such as oxidized LDL. Resveratrol inhibits AMMPRIN via suppression of ERK1/2 and p38 MAPK pathways (Huang et al. 2008).

Resveratrol, 1 mg/kg body weight per day, given from 6 weeks to 5 months of age, either alone or in a neutraceutical mixture containing various phytochemicals such as quercetin and inositol hexaphosphate (Longevinex®), led to a change in gene expression profile in mouse cardiac tissue similar to that seen after CR for this period (Barger et al. 2008). In all, expression of 630 genes changed in a similar direction in response to resveratrol and the neutraceutical mixture, although the latter affected 2,406 genes. One was FOXO1, which was not upregulated by resveratrol. This is consistent with quercetin mediating the effect on FOXO1. The latter then activates peroxisome proliferator-activated receptor α coactivator (PGC-1α) which interacts with mammalian target of rapamycin (mTOR). The PGC1α-mTOR complex is required to maintain mitochondrial function. Sirt1 protein was decreased in this work, although an increase in Sirt1 activity is seen in response to resveratrol (Baur et al. 2006; Lagouge et al. 2006). Each of the three treatments led to changes in cytoskeletal maintenance pathways, whereas CR uniquely affected several immune function pathways, and resveratrol uniquely affected several stress response pathways, but not pathways for inflammation and oxidant activity. Long-term CR and the neutraceutical mixture affected multiple metabolic pathways such as ones associated with glucose and lipid metabolism, oxidative phosphorylation and chromatin assembly. FOXO1 was a critical mediator of these effects.

In doses of 10 and 50 mg/kg body weight resveratrol is an efficient antihypertensive agent in a hemi-nephrectomy model of hypertension in rats (Liu et al. 2005). An antihypertensive effect has also been seen in obese Zucker rats given a daily dose of 10 mg/kg in water, systolic blood pressure being reduced to normal after a month (Rivera et al. 2009). The antihypertensive effect is likely to involve, at least in part, the increased eNOS expression and thus NO generation in blood vessel walls. In the spontaneously hypertensive rat (SHR), resveratrol reduced arteriolar wall thickness and antioxidant status, and increased vessel caliber (Chan et al. 2008b). The increase in resistance of blood vessels to oxidative stress involves upregulation of catalase, glutathione peroxidase and heme oxygenase-1 (Ungvari et al. 2007). Resveratrol targets quinone reductase type 2 in vascular smooth muscle cells to inhibit vascular smooth muscle proliferation (Cai et al. 2008). It also inhibits hypertrophy of cardiac myocytes (Chan et al. 2008) and does so by activating AMP-activated protein kinase (AMPK) via the AMPK kinase LKB1, and inhibition of protein kinase B/Akt. Although resveratrol could prevent cardiac hypertrophy and contractile dysfunction in the SHR, it was unable to lower blood pressure (Thandapilly et al. 2009).

Multiple pathways therefore mediate the beneficial effects of resveratrol and other polyphenols found in red wine on cardiovascular health. Since the effect of these is to protect against hypertension, ischemic heart disease, ischemic damage during myocardial infarction, and brain damage following cerebral ischemia, it is not surprising that an inverse association has been seen between dietary flavonoid consumption and cardiovascular mortality (Baur and Sinclair 2006; Perez-Vizcaino et al. 2006). The amount of resveratrol in red wine itself is relatively low and any small benefits it might have are offset by the toxic effects of ethyl alcohol present.

9.2.3 Diabetes

In streptozotocin-induced diabetes in rats resveratrol decreased insulin secretion, stimulated glucose uptake by hepatocytes, adipocytes and skeletal muscle, hepatic glycogen synthesis, and delayed insulin resistance (Su et al. 2006). In a rat model of obesity and type 2 diabetes, resveratrol (10 mg/kg body weight, p.o., given for 8 weeks) reduced insulin, improved lipid and inflammatory profile, lowered leptin, increased adiponectin, reduced TNF-α, increased aortic eNOS, and had an anti-hypertensive effect (Rivera et al. 2009). In diabetic rats resveratrol ameliorates kidney dysfunction and oxidative stress, as well as preventing diabetic nephropathy (Sharma et al. 2006). Resveratrol is a potent activator of AMPK, a sensor of cellular energy change, where AMPK phosphorylates and inhibits acetyl-CoA carboxylase, to increase fatty acid oxidation and inhibit fatty acid synthesis. Phosphorylation of AMPK accompanied Sirt1 activation by resveratrol and quercetin (Suchankova et al. 2009). Resveratrol thus offers potential benefit in prevention of type 2 diabetes, as well as in treatment of this disease.

9.2.4 Osteoporosis

Resveratrol exhibits anti-osteoporotic effects similar to hormone replacement therapy and the bone protective effect involves FOXO activation (Su et al. 2007).

9.2.5 Arthritis

The anti-inflammatory effects of resveratrol have been discussed above under other disorders associated with inflammation. Resveratrol dampens inflammation in arthritis and immune responsiveness in autoimmune diseases that include rheumatoid arthritis (reviewed by Harikumar and Aggarwal (2008); Magrone et al. (2008)). Resveratrol is a potent inhibitor of Cox-2 and NFκB and influences metabolism of arachidonic acid and eicasonoids to favor the anti-inflammatory, as opposed to inflammatory, products of these pathways. In human articular chondrocytes stimulated with the pro-inflammatory cytokine interleukin (IL)-1α, resveratrol inhibits the expression of VEGF, MMP-3, MMP-9 and Cox-2; its suppression of apoptosis and inflammatory signalling involves the NF-κB pathway (Shakibaei et al. 2008). Resveratrol also protects against inflammatory arthritis in a rabbit model (Elmali et al. 2007). This involved suppression of TNF-α and IL-1α induced NF-κB activation. In fibroblast-like synoviocytes from rheumatoid arthritis patients, resveratrol activates caspase-8, leading to apoptosis (Byun et al. 2008). Resveratrol, and other plant phytochemicals, thus have considerable potential in prophylaxis and treatment of arthritis (Khanna et al. 2007).

9.2.6 Neurological Disorders

Resveratrol can penetrate the blood-brain barrier to exert strong neuroprotective effects even at low doses of 0.1–1 μg/kg body weight i.v. (Baur and Sinclair 2006). It might therefore be useful in the treatment and prevention of neurological disorders such as Alzheimers disease, Parkinsons disease and Huntingtons disease (Anekonda 2006). Alzheimers disease is a progressive age-related, neurodegenerative disorder, characterized by the presence of neurofibrillary tangles and extracellular amyloid beta plaques in the cortex and hippocampus, areas of the brain that are important for memory and learning. By activating Sirt1, resveratrol protects against axonal damage in vivo and in vitro (Araki et al. 2004). Sirt1 activation, which inhibits NF-κB signalling, explains the protective effect of resveratrol against microglia-dependent beta amyloid toxicity (Chen et al. 2005). By repressing p53 it prevents apoptotic neuronal death, and by suppressing FOXO proteins it promotes neuronal survival.

Benefits to neuronal function are evident from an improvement in motor coordination and traction force conferred by resveratrol (Lagouge et al. 2006). In the Tg2576 model of Alzheimers disease, a grape seed polyphenol extract (MegaNatural-AZ) attenuated cognitive deterioration and amyloid deposition (Wang et al. 2008) by a potent inhibitory effect on fibril formation (Ono et al. 2008). It did so via inhibition of protofibril formation, pre-protofibrillar oligomerization, and initial coil→helix/sheet secondary structure transitions. The extract also had a protective effect in assays of cytotoxicity (Ono et al. 2008). A role for the resveratrol present is likely. These findings have obvious therapeutic implications.

In mouse models of Alzheimers disease and amyotrophic lateral sclerosis resveratrol reduced neuronal cell death and neurotoxicity of the mutant proteins expressed in these, namely p25 (a toxic coactivator of CDK5) and a mutant form of superoxide dismutase 1, respectively (Kim et al. 2007). It reduced neurodegeneration in the hippocampus and rescued associative learning in p25 mice. The effect of resveratrol in this study was mediated by SIRT-1, via increased deacetylation of p53 and/or PGC-1α. Earlier work had found that PGC-1α has a role in neuronal metabolism (St-Pierre et al. 2006), and the link between resveratrol, Sirt1, and PGC-1α was discussed above. The repression of p53 by resveratrol would likely protect neurons from oxidative damage and prevent apoptotic neuronal death. Increased neuronal survival might also involve suppression or activation of FOXO proteins, as supported by work in models of Huntington's disease caused by mutant polyglutamines in transgenic mice and *Caenorhabditis elegans* in which resveratrol rescued the phenotype of early neuronal dysfunction (Parker et al. 2005). As well, resveratrol, administered to mice, increased MnSOD protein level and activity in brain and this was independent of mitochondrial proliferation (Robb et al. 2008).

Another potential contributor to Alzheimers is the accumulation of localized neuronal damage surrounding tiny hemorrhagic mini-strokes within the brain (Cullen et al. 2006). The general cardiovascular benefit of resveratrol would, in the brain, therefore help protect against Alzheimers disease.

Resveratrol also protects neurological functions in events such as brain ischemia, stroke, seizure and epilepsy (Anekonda 2006). It therefore offers promise in the treatment and prevention of Alzheimers disease, Parkinsons disease, Huntingtons disease and other neurological disorders (Anekonda 2006).

9.2.7 Benefits to Other Organ Systems

Resveratrol confers benefits to many organs in the body (see review (Baur and Sinclair 2006)). It offers a renal benefit by protecting against ischemia reperfusion injury and carcinogen-induced DNA damage. It also has benefits in spinal cord trauma. In the liver it reduces inflammation, as well as cytokine production and thus increased vascular permeability. It protects the lungs, intestine and colon against harmful effects of toxins. It can protect against hearing loss. It also has analgesic effects. The list of benefits is not a complete one and will continue to grow.

9.2.8 Resveratrol Prevents Diseases of Obesity and Shortened Lifespan in a Mouse Model

In a mouse model of obesity, David Sinclair's group at Harvard University showed that resveratrol, 5 and 22 mg/kg body weight per day given to middle-aged mice for a year, was able to extinguish most of the adverse effects on health caused by a high-fat, high-calorie diet, the effect being more pronounced at the higher dose. The mice showed a marked reduction in cardiovascular disease, atherosclerosis, diabetes, cancer, hepatic steatosis (fatty liver), exhibited improved insulin sensitivity and motor function, lower insulin-like growth factor-I (IGF-I), and they lived to a normal mouse age (i.e, their lifespan was not decreased by 30% as is seen normally in obese mice) (Baur et al. 2006). This was not due to a weight-lowering effect of resveratrol. In fact the mice remained obese, and had high cholesterol, despite improved hepatic glucose and lipid metabolism. Subsequent studies have, nevertheless shown an ability of higher doses of resveratrol to reduce body weight in middle-aged obese mice.

Another striking discovery was that resveratrol, at a dose of 100 mg/kg body weight, fed to young mice for 3 months, made the mice resistant to diet-induced obesity, i.e., unlike the Harvard study that used doses 5- and 20-fold lower from middle age (Baur et al. 2006), the mice stayed lean despite being fed a high calorie diet, and an increase in their aerobic capacity was seen (Lagouge et al. 2006). More on this later.

In these studies resveratrol, by stimulating Sirt1, led to deacetylation, and thus activation, of PGC-1α (Baur et al. 2006; Lagouge et al. 2006) (Fig. 9.3). PGC-1α is an important regulator of mitochondrial function, energy balance and mitochondrial biogenesis all of which were enhanced in these mice. Consistent with enhanced muscle performance, the proportion of slow-twitch/oxidative muscle fibres was increased at the expense of fast-twitch/glycolytic fibres, and genes for oxidative phosphorylation were upregulated, as were mitochondrial genes (Lagouge et al. 2006).

The activation of PGC-1α that occurred in liver would normally increase gluconeogenesis, but the lowering of glucose observed was attributed to activation by resveratrol of AMPK, which potently inhibits gluconeogenesis. AMPK would also protect against fatty liver, because it increases fatty acid oxidation by inhibiting acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid synthesis. AMPK senses changes in stress and energy (low glucose) and can be activated directly by polyphenols (Zang et al. 2006).

The Harvard and French studies in 2006 highlight a parallel between the Sirt1 and AMPK signalling pathways (Koo and Montminy 2006). Each are triggered by fasting, and are energy sensing in that they respond to changes in NAD^+ and AMP. And each can increase lifespan. Both increase glucose utilization and enhance insulin sensitivity. Each pathway converges on PGC-1α and possibly other regulators of glucose and lipid metabolism.

A reduction in core body temperature is associated with reduced energy expenditure and improved survival in mice, and vice versa (Conti et al. 2006), yet the increased fat metabolism by the mitochondria-rich brown fat leading to more adipose tissue burn off suggests that the higher dose of resveratrol used in the Auverx study was sufficient to trigger Sirt1 activation (Koo and Montminy 2006). The 10–20 fold lower doses of resveratrol in the study by the Sinclair group may explain why core body temperature did not increase in their mice and why their mice were not protected from obesity (Koo and Montminy 2006).

9.2.9 Genome-Wide Changes in Gene Expression in Response to Resveratrol

Each study of obese mice above carried out whole genome expression profiling. This revealed that genes relevant to the changes seen in each tissue were induced (Baur et al. 2006; Lagouge et al. 2006). An outline of the findings follows.

9.2.9.1 Liver

The Harvard group studied liver. In this tissue, the obese mice showed a change in expression of 1.9% of genes (Baur et al. 2006). The gene *Cidea*, that regulates energy balance in brown fat and is obesogenic and diabetogenic, was the most upregulated gene in the obese mice, and was most potently downregulated in the resveratrol-fed group (Baur et al. 2006). Resveratrol affected 127 pathways. These included pathways involved in the tricarboxylic acid cycle, glycolysis (a well-known marker of calorie restriction), complement, butonate and propanoate metabolism, sterol biosynthesis and Stat3 signalling. The latter, which was upregulated, is a transcription factor involved in cell survival and liver regeneration. Stat3 in liver is suppressed by a high calorie diet and shows a decline with age, an effect ameliorated by CR. In all, resveratrol opposed the effect of the high calorie diet in the case of 144 pathways out of the 153 that were altered significantly in their mouse model of obesity (Baur et al. 2006). The gene expression profile of the obese mice fed resveratrol resembled much more closely that of mice on a standard diet than mice on the high calorie diet. Overlap was seen for 19 of 36 pathways altered by CR. Those that changed in the same direction for both resveratrol treatment and CR included IGF-I and mTOR signalling, glycolysis (each downregulated), and Stat3 signalling (upregulated). Pathways that changed in opposite directions for each were cell cycle checkpoint and apoptosis (elevated in CR, but lowered by resveratrol).

9.2.9.2 Muscle

The French group performed gene expression profiling on muscle tissue. They found 30 gene sets that were enriched significantly after resveratrol treatment. These included ribosomal RNA processing, striated muscle contraction, electron transport chain, oxidative phosphorylation and ATP synthesis. Genes increased were concerned with muscle contraction, such as troponins, as well as those that could increase oxidative metabolic status, such as components of the respiratory apparatus, oxidative enzymes, and ATPases that could provide a slow, stable long-lasting supply of ATP for muscle contractions and elevated endurance. Also enriched were transcripts of genes for organelle (mitochondrial) biogenesis such as RNA-processing enzymes and ribosomal subunits. All of this explains the increased oxidative capacity of muscle seen in the resveratrol-treated mice.

A significant increase in PGC-1α was seen and would have contributed to the increased expression of genes for oxidative phosphorylation in muscle (Lagouge et al. 2006). A marked increase in a downstream target of PGC-1α, oestrogen-related receptor (ERR), was seen, as was an ERR/PGC-1α target, nuclear respiratory factor-1, and the target of the latter, mitochondrial transcription factor A, that is crucial for expression of important genes in mitochondrial DNA. Also elevated were other targets for PGC-1α, including genes for fatty acid oxidation, such as medium chain acyl-CoA dehydrogenase, uncoupling and protection against ROS, such as uncoupling protein 3, and myoglobin and troponin 1. An increase in peroxisome proliferators-activated receptor (PPAR) was seen, and this is known to induce genes that facilitate β-oxidation of fatty acids. Higher uncoupling protein-1 would contribute to uncoupling of respiration from ATP synthesis to produce heat in brown adipose tissue. Interestingly, no effect was seen on mitochondrial gene expression in the heart consistent with lack of effect of resveratrol on heart physiology. Also no effect on acetylation of PGC-1α was seen in heart, but an increase in PGC-1α deacetylation was observed in other muscles. This and other experiments pointed to an important role for Sirt1 in mediating the effects of resveratrol on PGC-1α expression. Resveratrol also improved insulin sensitivity.

9.2.10 Effect of Resveratrol in Normal Mice

When the Harvard group, together with de Cabo's group at the National Institutes of Aging, tested the effect of resveratrol in normal mice (which required a higher dose of 100 mg/kg body weight/day in order to achieve plasma levels comparable to those in the fat-fed mice), only a trend to increased lifespan was seen (Pearson et al. 2008). Every other day feeding (a form of calorie restriction) did not increase lifespan by itself, but did, significantly, when resveratrol was included (Pearson et al. 2008). The authors, at the National Institute of Aging and Harvard, have subsequently gone on to begin resveratrol at weaning rather than middle age, and a more pronounced effect on lifespan is anticipated. Even though resveratrol slows the general decline in health with aging, it does not suppress lymphoma, which is in mice a major cause of mortality. Therefore, could resveratrol have a greater effect in humans than in mice?

There was an improvement in cardiovascular function, bone density and motor coordination (Pearson et al. 2008). This was accompanied by a general decrease in oxidative stress and inflammation (Pearson et al. 2008). Most notably, in the aorta, the researchers saw a decrease in superoxide production and a reduction in gene transcripts for inflammatory processes. A reduction in cataracts also pointed to reduced oxidative stress.

9.2.11 Exercise Performance

In the French study above, it was found that muscles burnt more energy, worked more efficiently, and the mice treated with resveratrol could run twice as far before exhaustion (Lagouge et al. 2006). One implication is that resveratrol might be a new performance-enhancing "drug". Not surprisingly, this work has attracted the attention of sporting bodies such as the International Olympic Committee (IOC). No change in liver histology was observed in the study, but this could have been contributed by their younger age and relatively shorter treatment with resveratrol compared with the Harvard study.

A PPARα agonist and exercise training has been found to increase oxidative myofibers synergistically and enhance endurance in mice in a treadmill running test (Narkar et al. 2008). Exercise training activates AMPK and PGC-1α. In an astounding experiment, giving an AMPK agonist, AICAR, was able to induce metabolic genes and increase running endurance by 44% in sedentary mice, so demonstrating the potential for orally active drugs that can boost performance without the need for exercise (Narkar et al. 2008). There is no doubt that this too will interest the IOC.

The clinical potential is of great theoretical interest. It is well-known that the various common chronic conditions of aging are associated with lack of adequate physical activity. Exercise helps protect against these conditions. It does so in part by suppressing inflammation, which is a feature seen in atherosclerosis, neurological disorders, type 2 diabetes and cancers (reviewed in Handschin and Spiegelman (2008)). Thus by activating PGC-1α, such as by resveratrol, the latter diseases, as well as muscle wasting, sarcopenia and muscular dystrophies, could be prevented (Handschin and Spiegelman 2008) (Fig. 9.3). A similar benefit might result from administration of the AMPK agonist, AICAR.

9.2.12 Aging

We now have an enormous understanding of aging at the molecular level, and of the pathways that when manipulated can increase the lifespan of experimental species (reviewed by Vijg and Campisi (2008)). Resveratrol has been shown to increase the lifespan of all species tested. The effect is strongest for short-lived species. An unanswered question is whether resveratrol or related compounds might lengthen human lifespan?

Resveratrol itself is metabolized rapidly. So could it, or its metabolites, have beneficial effects at doses that might be achieved in vivo? In recent times promising results have been obtained in mammals, mostly mice. Although polyphenolic compounds such as resveratrol mimic the effect of CR on health and lifespan of non-human species tested, CR results in a significant increase in lifespan, whereas the effect of resveratrol is modest at best. Anyone who caloric restricts themselves by the 30–40% that increases rat lifespan feels that they are living on the brink of starvation continuously, and so is challenging for most humans. In contrast, if it works, taking a pill would offer a desirable alternative by activating similar pathways in order to achieve improved "health-span", and possibly, lifespan.

Figure 9.3 outlines common aspects of the pathways activated by CR and resveratrol (and other polyphenols) (Wood et al. 2004; Morris 2005). As discussed in the preceding chapters and various reviews (Kwon and Ott 2008), an early target of each of these is the sirtuin class of NAD$^+$-dependent deacetylases. Resveratrol binds within the active site of SIRT1 and lowers the K_m by up to 35-fold (Howitz et al. 2003). Whether the activation of sirtuins by resveratrol in vivo is direct, or is indirect, via activation of AMPK that then results in an increase in the sirtuin activator NAD$^+$, is controversial (Canto et al. 2009). One action of sirtuins is activation or suppression (depending on cell type and circumstances) of the FOXO group of transcription factors. FOXOs target acceptor sites in the promoter DNA of various genes, leading to increased or decreased transcriptional activity, depending on the gene. The change in mRNA profile of the cell means an alteration in concentration of particular proteins. Many of these are enzymes. The result can include a decrease in apoptosis, an increase in antioxidant activities, DNA protection, anti-inflammatory effects, and various other changes that foster good cellular health, and thus a healthier profile of the organism (reviewed by Burgering and Kops (2002); Morris (2005); Gross et al. (2008)). In other organisms at least, a major result of stimulation of these sirtuin pathways is to ramp up stress resistance pathways in cells and so increase survival in times of adversity (Guarente and Picard 2005). The sirtuin enzymes, and the effect of polyphenols on them, have been conserved through evolution. Their role in stress resistance has no doubt expanded over time and speciation as new stresses and demands have emerged, so explaining the similar effects they have in diverse species (Koubova and Guarente 2003).

The result of overexpression of sirtuins in cultured cells is similar to that of resveratrol treatment (Howitz et al. 2003). Sirt1 activation leads to deacetylation of p53 (Howitz et al. 2003). Suppression of p53 delays apoptosis, allowing cells more time to repair damage, and also prevents needless cell death (Howitz et al. 2003). In human HEK 293 cells, Sirt1 activation by 0.5 μM resveratrol increased survival of cells after exposure to ionizing radiation (Howitz et al. 2003). The mechanism included suppression of Bax-mediated apoptosis (Cohen et al. 2004). Observations at the cellular level appear relevant to the organism, as in each case survival and longevity are linked intimately.

After exposure of human foreskin fibroblasts in culture to resveratrol my Lab identified 47 genes whose expression was altered 2-fold or more (Stefani et al. 2007). Many of these were in pathways that affect stress-resistance and cell lifespan. Two that were suppressed were in the *Ras* (cancer) pathway. Resveratrol also suppressed expression of the senescence marker INK4a (now termed CDNK2A) (Stefani et al. 2007) that is involved directly in the aging process (Janzen et al. 2006; Krishnamurthy et al. 2006; Molofsky et al. 2006). Resveratrol, by activating sirtuins, might stimulate the shuttling of cytoplasmic (inactive) FOXOs to the nucleus, where they activate genes for stress-resistance and other pathways that assist in cell survival (Stefani et al. 2007).

One of the earliest observations was that in yeast, Sir2, the homolog of mammalian Sirt1, reduces aging by relocalizing to sites of genomic instability, in particular by suppressing recombination at the ribosomal DNA (rDNA) locus that gives rise to toxic rDNA circles (Sinclair and Guarente 1997). Now a similar process has been discovered in mice (Oberdoerffer et al. 2008). Sirt1 suppressed repetitive DNA in mouse embryonic stem cells and affected expression of a diverse array of genes across the mouse genome. When DNA damage occurs, Sirt1 dissociates from the sites of repetitive DNA and relocalizes to DNA breaks in order to assist in repair. In a mouse model of genomic instability, *SIRT1* expression aided in survival, in part by suppressing the transcriptional alterations that occur with aging (Oberdoerffer et al. 2008). These findings provide a direct link between DNA damage and changes in gene expression with aging. Resveratrol, by activating Sirt1, may promote genomic stability, so as to delay aging. Moreover, resveratrol and other dietary constituents could potentially influence the quality of stem cells, possibly via the mother's diet in utero, consistent with the Barker hypothesis, interpreted in broad terms (Trosko 2008).

9.2.13 Dose and Metabolism

The first human known to attempt CR was Luigi Cornaro in the 16th century, who included 14 oz. of red wine in his daily diet. Whether the resveratrol present contributed to his long life might be debated though. In animals, claims of beneficial effects of resveratrol span a range of doses from 0.1 to 1,500 mg/kg body weight (Baur and Sinclair 2006). After oral ingestion by humans, resveratrol is absorbed rapidly, with a half-life of only 8–14 min (Walle et al. 2004). At least 70% of a dose of 25 mg is absorbed by the gut (Walle et al. 2004). Most is converted to sulphate conjugates in the liver or intestine within 30 min and this limits its bioavailability. Its metabolites, however, have a serum half-life of 9.2 h. After ingestion of a radioactively labelled dose, 53–85% of the radioactivity can be found later in urine (Walle et al. 2004). Following consumption of 25 mg by a 70 kg adult, serum resveratrol reaches 20–40 nmol/L, whereas its derivatives reach approx. 2,000 nmol/L (Yu et al. 2002). It has been suggested that its metabolites might retain or mediate at least some of the beneficial effects of resveratrol (Baur and Sinclair 2006).

In the case of extracts of various grape vine cultivars, potency varies considerably (Räthel et al. 2007). Assuming an optimistic estimate of 5 mg/L for the concentration of resveratrol in red wine, two glasses (375 mL) would provide a dose of 27 μg/kg in a 70 kg person (Baur and Sinclair 2006). This would lead to a peak serum concentration of approx. 2.4 nM for free resveratrol and 180 nM for metabolites. After consumption of 375 mL of red wine daily for 2 weeks plasma polyphenol content reaches approx. 450 μg/mL (Nigdikar et al. 1998). Such a level might provide cardiovascular benefits by increasing NO in the blood vessel wall (Leikert et al. 2002), but may be too low to confer other benefits. For ingestion of a pharmacologically relevant dose (by direct translation to humans of data for mice based on body weight), such as 100 mg/kg per day, serum levels would be 9 μM authentic resveratrol and 680 μM total resveratrol (Baur and Sinclair 2006). Tissues accumulate resveratrol (Vitrac et al. 2003). It is, however, noteworthy that resveratrol works in synergy with other dietary compounds such as quercetin, ethanol, vitamin E and catechins (Wallerath et al. 2005) and such interactions might contribute to the beneficial effects of low doses such as are found in red wine and other dietary sources (Baur and Sinclair 2006).

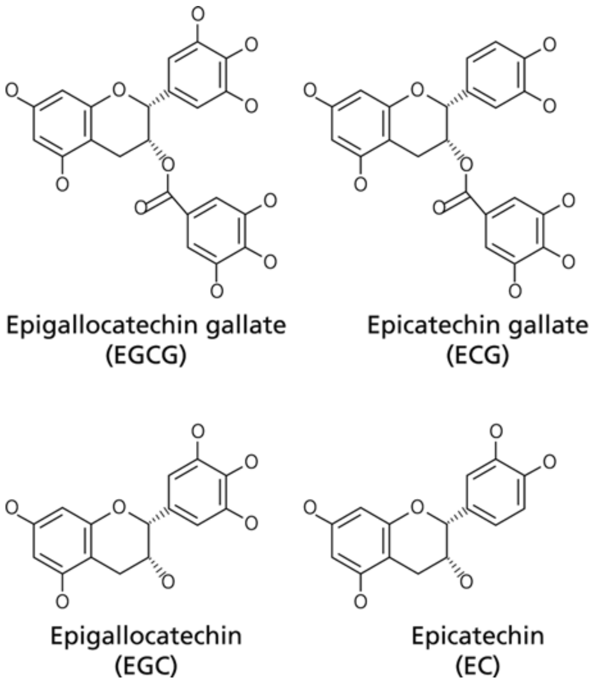
In pure form resveratrol is safe at doses up to 300 mg/kg body weight (Baur and Sinclair 2006). In mice, a dose of 18 g /kg body weight per day is accompanied by the deaths of most mice within 3–4 months (Pearson et al. 2008). A similar toxic effect has been seen with such very high doses in rats (Crowell et al. 2004).

There are numerous commercial sources of resveratrol. One manufacturer, Orchid Chemicals & Pharmaceuticals Ltd, Chennai, India (distributed by LaliLab, Durham, NC, USA at a cost of US$415 per 100 g satchel), provides laboratory-grade resveratrol. If one were to consume 1 g/day, this would represent a 3 month supply and would cost US$138 per month, excluding shipping. A published cost estimate, for taking 100 mg/kg body weight per day (= 7 g/day for a 70 kg human) was US$600 per month (Baur and Sinclair 2006). It has, however, been argued that translation of dose from animals to humans should involve a formula based on body surface area normalization, not body weight (Reagan-Shaw et al. 2007). This reduces the dose of resveratrol 10-fold, so that 1 g/day becomes 100 mg/day. It should be noted that many preparations become depleted of activity by the time they reach the consumer. It is therefore desirable to ensure the supplier ships on dry ice, and that the resveratrol is stored in the freezer and protected from light.

9.3 Catechins

Other flavonoids with health benefits include (–)-epigallocetechin-3-gallate (EGCG; from green tea), genistein (from soy products), curcumin (from the roots of turmeric), sulforaphane (found in cruciferous vegetables such as broccoli), and quercetin (found in apples and red onions). Just as resveratrol, they exert neuroprotective, cancer preventive and beneficial cardiovascular effects. Besides the long-held view that they work via antioxidative effects, they can induce apoptotic pathways, suppress cytochrome P450 enzymes, cell cycle progression and chromatin remodelling, inhibit angiogenesis and exert anti-inflammatory activity (reviewed in Dashwood et al. (2006); Mattson and Cheng (2006); Myzak and Dashwood (2006); Juge et al. (2007); Morris (2008)). Here just some of these will be outlined, starting with the catechins.

Fresh leaves from the tea plant (*Camellia sinensis*), and green tea in particular, contain high levels of types of flavonoids or flavonols referred to collectively as catechins. Most notable is EGCG (Fig. 9.5). ECGC, together with other polyphenols, represent 30–45% of solid extract of green tea (Wang et al. 1994). Catechins and their derivatives scavenge ROS directly. As well they activate transcription factors and antioxidant enzymes, so supplementing their direct effect (Higdon and Frei 2003). Antioxidant potency of tea catechins is: (–)-epicatechin-3-gallate (ECG) > (–)-epigallocatechin (EGC) > (–)-epicatechin (EC) (Guo et al. 1999). In addition catechins chelate metals; this involves the 3′,4′-dihydroxyl group in the B ring (Hider et al. 2001) and gallate group (Guo et al. 1996). As a result it neutralizes the ferric ion, which is converted to (inactive) iron, so protecting cells from oxidative damage (Grinberg et al. 1997). In these and other ways catechins increase cell survival (Mandel et al. 2005).



Epigallocatechin gallate (EGCG)

Epicatechin gallate (ECG)

Epigallocatechin (EGC)

Epicatechin (EC)

Fig. 9.5 Chemical structures of catechins and derivatives found in green tea

Consumption of 200–300 mg ECGC (or 5–6 cups of green tea) per day have significant cardiovascular, metabolic, and perhaps cancer benefits, discussed below.

9.3.1 Cancer

Prevention of cancer may be one important benefit of drinking green tea (Park and Surh 2004). For example, the lower rate of breast and prostate cancer in Asians has been attributed to EGCG consumption in tea (Park and Surh 2004). EGCG suppresses the proliferation of melanoma, breast cancer, lung cancer, leukaemia and colon cancer cell lines (Lambert and Yang 2003). Doses of ECGC 10 times higher than attained by drinking tea inhibit MAPKs, CDKs, growth factor-related cell signaling, activation of activator protein 1 (AP-1), NF-κB, topoisomerase I, MMPs, and other targets (Lambert and Yang 2003). ECGC also inhibits constitutive expression of Cox-2, known to play a critical role in colon cancer. This involves inhibition of the activation of NF-κB (Peng et al. 2006). EGCG binds to DNA and RNA (Kuzuhara et al. 2006). This might explain why EGCG protects DNA from damage induced by ROS, ionizing radiation and ultraviolet light, and from DNA methylation (Fang et al. 2003). The constituent hormetic compounds in green tea therefore result in molecular changes that inhibit cancer pathways in cells (Fujiki 2005).

9.3.2 Cardiovascular Disease

Perhaps the strongest benefits are to cardiovascular health (reviewed by Wolfram (2007)). Consumption of five or more cups of green tea per day compared with one or fewer cups reduced mortality from all causes dose-dependently over the 11 years of a Japanese study by 23 and 12% in women and men, respectively (Kuriyama et al. 2006). Cardiovascular disease was reduced by 31% in women, a reduction in stroke being the main effect (–37%), and particularly noticeable was cerebral infarction (–51%). No difference in cancer incidence was observed over the period of this study. Another Japanese study confirmed a reduction in subarachnoid hemorrhage (Okamoto 2006). Coronary atherosclerosis was reduced by 60% for Japanese men who drank 4 or more cups of green tea per day (Sasazuki et al. 2000).

Daily consumption of 600 mL of green tea per day reduced the risk of hypertension in Chinese subjects by 65%, with benefits of lesser magnitude seen for lower daily doses (Yang et al. 2004). The anti-hypertensive effects of tea flavonoids may include improvement in endothelial function (Hodgson 2006). Green tea and EGCG improve blood flow, increase antioxidant capacity and reduce oxidative damage (reviewed by Wolfram (2007)). Green tea (13 g/L p.o.), given to diabetic spontaneously hypertensive rats for 3 months, reduced body weight, lowered oxidative status by re-establishing the oxidative state, and reduced nephropathy, but did not affect glycemia or blood pressure (Ribaldo et al. 2008). Similarly, green tea extracts (6 mg/mL per day; 3.5 mg EGCG) prevented hypertension that is normally seen in rats given angiotensin II (Antonello et al. 2007). The treatment also prevented the increase in left ventricular mass index, as well as aortic media-to-lumen ratio, hydroxyperoxide radicals, heme oxygenase, p22^{phox}, and SOD mRNA.

Benefits seen in other studies of cardiovascular disease, including atherosclerosis, heart disease and hypertension, can be found in review by Wolfram (Wolfram 2007)

9.3.3 Diabetes

Green tea prevents type 2 diabetes (reviewed by Wolfram (2007)). For Japanese consuming six or more cups per day, risk reduction was 33% (Iso et al. 2006). The glucose-lowering (insulin-like) properties of green tea (Tsuneki et al. 2004) and EGCG (Kao et al. 2000) point to potential use as an antidiabetic agent. (For additional studies refer to Wolfram (2007).) EGCG and ECG inhibit glutamate dehydrogenase and can thereby modulate insulin secretion (Li et al. 2006). This effect involves induction of PI3K-mediated phosphorylation of insulin-sensitive residues on FOXO1a. The latter then represses enzymes, such as phosphoenolpyruvate carboxykinase, which is rate-limiting in gluconeogenesis (Anton et al. 2006). The induction by ECGC of FOXO1a is sensitive to ROS. An effect in improving plasma lipid profile, fat oxidation, exercise capacity, energy expenditure, and counteracting obesity has also been seen in various studies (reviewed by Wolfram (2007)).

9.3.4 Neurological Disorders

Like other flavonoids, catechins can help in the treatment of diseases involving amyloid plaque. EGCG reduces the misfolding and oligomerization of mutant huntingtin and this helps prevent neural defects that occur in transgenic *Drosophila* in which the mutant protein is overexpressed (Ehrnhoefer et al. 2006).

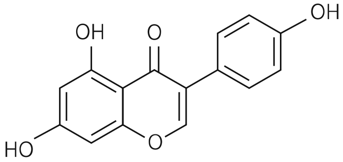
9.3.5 Lifespan

EGCG has also been shown to extend the lifespan of stressed nematode worms (Zhang et al. 2009).

9.4 Genistein

The most abundant isoflavone in soy products is genistein (4′, 5, 7-trihydroxyisoflavone) (Fig. 9.6). Concentrations in soy products range from 2 to 200 μg/g. Genistein helps to lower the risk of coronary heart disease and cancer (Cassidy 2003), and is safe, even at high concentrations such as those used in cancer trials (Bloedon et al. 2002; Busby et al. 2002). In this regard, trials of genistein for treatment of breast, prostate and uterine cancers, as well as osteoporosis, cardiovascular disease and menopausal symptoms are underway.

Fig. 9.6 Structure of genistein



9.4.1 Cancer

Soy products in the Asian diet means consumption of genistein in such populations is high, so possibly accounting in part for their lower rates of breast and prostate cancer (Park and Surh 2004). Genistein also seems to increase the efficacy of radiation therapy used to treat these cancers (Ravindranath et al. 2004). Genistein protects melanocytes from melanoma induced by UV-B radiation and reduces melanoma incidence (Ravindranath et al. 2004). Part of this effect involves stimulation of melanin production and tyrosinase activity (Ravindranath et al. 2004).

This isoflavone is able to arrest cell growth and proliferation, as well as stop cell cycle progression at the G2/M stage, and suppress tumor invasion and angiogenesis (Sarkar and Li 2002; Ravindranath et al. 2004). The mechanism involves inhibition of the activities of protein tyrosine kinase, topoisomerase II and MMP-9, as well as by down-regulating the expression of at least 11 genes, including the VEGF gene (Ravindranath et al. 2004). In addition, genistein alters the expression of gangliosides and other carbohydrate antigens and this helps facilitate their immune recognition. It acts synergistically with tamoxifen, cisplatin, dexamethasone, and other drugs, as well as with other flavonoids such as quercetin, green-tea catechins and black-tea thearubigins (Ravindranath et al. 2004).

9.4.2 Cardiovascular Disease

Drinking soy milk for an extended period is able to lower blood pressure in mild to moderate essential hypertension (Rivas et al. 2002). Beneficial effects have been noted on the walls of blood vessels. At physiological concentrations in human vascular endothelial cells, genistein alters expression of genes whose products control blood pressure; e.g., endothelin converting enzyme-1, endothelin-2, oestrogen receptor and atrial natriuretic factor receptor A precursor (Ambra et al. 2006). Genistein also counters the effect of oxidized LDL on expression of VEGF receptor types 1 and 2.

9.4.3 Neurological Disorders

Benefits of relevance to neurological disorders include the ability of genistein to bind strongly and selectively to and inhibit transthyretin in plasma, as well as to disease-associated genetic variants of transthyretin (Green et al. 2005). This then reduces by over 90% the ability of each to induce amyloid fibril formation in vitro (Green et al. 2005). Senile systemic amyloidosis is characterized by the deposition of transthyretin in the heart and peripheral nerves. Therefore such an action might explain why genistein is an exceptional inhibitor of transthyretin amyloidogenesis.

9.4.4 Osteoporosis

An anti-osteoporotic effect is well known. Soy isoflavones have a mild, but significant effect on bone mineral content in postmenopausal women with low bone mass (Chen et al. 2003).

9.5 Novel Pharmaceuticals

Synthetic sirtuin activators and inhibitors have potential as pharmaceuticals in the treatment of cancer. Nicotinamide is a well-known inhibitor of sirtuins.

Sirtinol, splitomycon, various indoles and their derivatives, as well as the calchone polyphenol 3,2′,3′,4′-tetrahydroxylchalcone represent additional sirtuin inhibitors of possible clinical use (Kahyo et al. 2008).

Activators of Sirt1 have been discovered by screening of hundreds of thousands of chemicals (Milne et al. 2007). Some were a thousand-times more potent than resveratrol, which itself is the most potent natural Sirt1-activator (Milne et al. 2007). These bind to an allosteric site (amino acid residues 183–225) abutting the active site of Sirt1 to lower the K_m for acetylated substrates.

In mouse models of obesity they lower plasma glucose, improve insulin sensitivity and increase mitochondrial capacity, supporting their potential as novel therapies for type 2 diabetes. In that study the most potent was SRT1720. The latter enhanced endurance running performance, and had a strong protective effect against diet-induced obesity and insulin resistance by its ability to increase oxidative metabolism in skeletal muscle (Feige et al. 2008). The effect of SRT1720 in this study involved the deacetylation of PGC-1α, FOXO1 and p53, and indirect stimulation of AMPK signaling via a global network controlling fatty acid oxidation and mimicking low energy levels. More recently, a safe, potentially better, compound, STC2104, has been found after large-scale further screening of synthetic compounds by Sirtris Pharma and the Sinclair group at Harvard.

The medical potential offered by resveratrol has led to clinical trials for type 2 diabetes, in “raw” (never-treated) cases conveniently sourced in India. This and the more potent, synthetic sirtuin activators have enormous potential not just in pharmacotherapy for diabetes, but also for cardiovascular diseases, neurodegenerative disorders and certain cancers. They offer enormous potential for the prevention and treatment of many common life-threatening conditions. Apart from the improvement in “health-span” and thus average lifespan by prevention and treatment of these ills, resveratrol or related compounds may have the potential to lengthen average human lifespan, just as is seen in other species. This remains to be shown, however.

In *C. elegans* and mice a reduction in the activity of clock abnormal protein 1 (CLK-1) and its mouse homologue mclk1 (coenzyme Q7) slows down aging (Wang et al. 2009). CLK-1/mclk1 is a mitochondrial hydroxylase involved in ubiquinone (coenzyme Q) biosynthesis (Wang et al. 2009). The drug, clioquinol (5-chloro-7-iodoquinolin-8-ol), inhibits mammalian mclk1 activity in cultured cells, and this involves chelation of iron and cobalt cations (Wang et al. 2009). As reviewed in Wang et al., slowing down of the aging process may explain why clioquinol has been seen to exhibit benefits in animal models of neurodegenerative diseases, in which its action includes chelation of metal ions. In cancer it stimulates TNF-α secretion.

Rapamycin, a macrolide antibiotic, by inhibition of mTOR, a downstream mediator of growth in the insulin signaling pathway, prolongs the lifespan of middle-aged mice by 28% for males and 38% for females (Harrison et al. 2009). Treatment with a commonly used anti-hypertensive drug, enalapril, an angiotensin converting enzyme inhibitor, reduces mortality of high-fat fed rats by 45% (Santos et al. 2009)

9.6 Conclusions

The information accumulated to date on natural polyphenols indicates their considerable benefit to health. Even though chemicals such as resveratrol are metabolized rapidly, their effects are profound. As a result, all of these, as well as the newer synthetic sirtuin activators, offer considerable appeal as dietary supplements, even though CR is more effective. This is because CR is challenging for most people, whereas taking a pill is easy, and many of the same beneficial pathways are activated.

From a compendium of over 35,000 species of vegetables, fruits and nuts one can find more than 4,000 different flavonoids (Howes and Houghton 2003; Howes et al. 2003). So it can be appreciated that what is known so far is merely a little about some of the most well-known and best researched compounds. As indicated in Table 9.1, there are thousands of published studies. In the present chapter I have covered only a handful of these. There may be other natural chemicals that turn out to be even more important or potent. Nevertheless, from what is known, the benefits of the various polyphenols include both overlapping features as well as some distinct properties. Cataloging them all presents a massive task. A common perception is that plant-derived compounds, being natural, are safer than synthetic drugs. Of course one must not forget that the plant world contains many highly toxic chemicals as well, so thorough research and testing is needed to evaluate each one. In the USA, the dietary supplements industry is a $7 billion plus per annum industry (Glaser 1999) and worldwide exceeds $30 billion (Raskin et al. 2002).

Whereas the ability of conventional pharmaceuticals to target specific molecules (i.e., their specificity) has been seen as desirable, the downside is that they often have side effects, may be ineffective or partially effective in some patients, and are generally expensive. In contrast, the broad effects of natural chemicals and their lack of side effects at therapeutic doses is now forcing a reconsideration of the “specificity” concept. The most intensely studied, resveratrol, affects numerous important pathways in the cell. It does so by binding to various signaling molecules and activating several transcription factors. This leads to alteration in the expression of proteins involved in cell cycle control, apoptosis, kinase effects, inflammation, angiogenesis and metastasis. While the safety of natural polyphenols, especially when present in raw food is virtually assured, the advent of more powerful synthetic molecules that act on similar intracellular targets offers hope, once safety is proven, for the treatment of cancer, cardiovascular diseases, type 2 diabetes, neurological disorders, autoimmune diseases, skin disorders, arthritis and the aging process itself.

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Chapter 10

Will Calorie Restriction Stave Off Age-Related Brain Dysfunction, Specifically to Learning and Memory? A Review and Critique of the Rodent Literature

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10.1 Introduction

Experimental studies spanning seventy plus years (McCay et al. 1935) have revealed that consuming significantly less calories (30–50% less than ad libitum) while providing supplemental micronutrients and vitamins is beneficial to both health and lifespan in a wide variety of species. In fact, calorie restriction (CR) remains the only non-genetic intervention known to extend mean and maximum lifespan in both mammalian and non-mammalian species, slow age-related decline in nearly every organ studied, and improve function on a cellular and molecular level (Masoro 1993). CR initiated in early adulthood seems to be most beneficial to health and lifespan, although benefits have been observed when CR was initiated at mid-life and even in senescence (Takahashi and Goto 2002). Regardless of the manner in which the restriction occurs, reducing overall calorie consumption and not individual macronutrients seems to be the critical factor that confers these health and longevity benefits (Weindruch and Walford 1988; Table 10.1).

The mammalian brain controls a complex array of bodily functions through equally complex signaling pathways (Mattson and Lee 2002; Russell and Kahn 2007). Therefore, it is not surprising that the central nervous system requires considerably more energy to function compared to other organ systems (Freemantle et al. 2006) and current dietary guidelines recommend intake of 130 g of carbohydrate daily solely to fuel the activities of the brain and CNS. This is a significant portion of total calorie intake, and though the human brain represents only about 2% of total body weight it receives about 15% of the cardiac output, accounting for about 20% of total body oxygen consumption, and roughly 25% of total body glucose utilization (Magistretti et al. 2000). Given the highly energetic nature of the brain, it is

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Table 10.1 Typical CR diets for rats and mice

Calorie Restriction	Fed 10–50% less than daily consumption of paired ad libitum (AL) controls with supplementation of vital nutrients (vitamins, minerals)
Every Other Day (EOD)	Provided food AL every other day
Hypocaloric Diet	AL fed diet that consists of 30–50% less calories. Diet often contains fillers such as fiber.

logical to hypothesize that conditions which restrict energy, like CR, would have potent effects on brain activity and function.

Indeed, the number of studies documenting the positive effects of CR on brain aging has steadily increased over the last three decades (Mattson and Lee 2002; Gillet-Guyonnet and Vellas 2008; Contestabile 2009). However, CR's effects on brain function, and specifically those on learning and memory in aged rats and mice, are not all positive (May et al. 1992). In fact, some investigators have argued against employing CR as an intervention for learning and memory enhancement (Vitousek et al. 2004), not only because of inconclusive results but also out of a concern for the potential development of genuine disordered eating characterized by chronic, severe under-eating in the absence of proper nutritional supplementation – anorexia nervosa.

It seems clear that CR does improve learning and memory in young and middle aged animals as there are numerous studies demonstrating these benefits (Wu et al. 2003; Hashimoto and Watanable 2005; Martin et al. 2007; Fontán-Lozano et al. 2008). However, these studies, while alluding to benefits for aging, do not answer the question as to whether long-term CR will benefit brain function in the aged, especially learning and memory. Clearly, the only way to answer this question is to assess the effects of CR on the aged animal. In an effort to better understand this particular question, the remainder of this chapter will focus on existing studies that utilize senescent animals with the intent of providing further insight on the potential benefit of long-term CR on behavioral outcomes of learning and memory studies.

In the following sections the effects of early- and mid-age onset of CR on learning and memory in aged rats and mice are summarized. We begin by discussing the benefits of long-term CR on brain aging. Next we review some of the methodologies (and associated confounding issues) employed in the study of aging, learning and memory in rodents on CR, and follow this with a third section dedicated to a more thorough evaluation of the behaviors observed in learning and memory studies in aging rat and mouse populations on CR. A final concluding section is dedicated to further interpretation of the findings to date, issues that need to be further clarified and studied, and our opinion on the direction research on dietary interventions to prevent age-related loss in mnemonics should take.

10.2 Brain Aging

With pathological aging, numerous changes detrimental to learning and memory occur in the brain. Alzheimer's disease (AD) and Parkinson's disease (PD) are two age-related neurodegenerative disorders in humans that cause a decline in learning and memory and ultimately lead to death. In these diseases, cellular damage and death occur in brain areas that are involved in learning and memory, particularly the hippocampus in AD and the substantia nigra in PD. Likely mechanisms for cellular damage and death in brain tissue include oxidative damage and inflammation. CR seems to be able to slow the accumulation of damage occurring from these mechanisms and appears to condition cells to protect them from a multitude of stressors including neurotoxins (Prolla and Mattson 2001; Joseph et al. 2005). CR also seems a likely candidate as an intervention for diabetes and stroke, two common conditions in aging that contribute to increasing numbers of individuals with mnemonic loss (Mattson 2000).

A summary of some of the documented benefits of CR are displayed in Table 10.2. On a cellular level CR provides increased resistance to stress to the aging brain particularly from oxidative stress and inflammation (Joseph et al. 2005). In addition, CR seems to make neuronal cells more resistant to programmed cell death, or apoptosis (Mattson and Lee 2002). Specific mechanisms that may confer these benefits from CR include an increase in brain-derived neurotrophic factor (BDNF, a growth factor that promotes neuronal cell development and survival) and reductions to amyloid plaque development. Regarding BDNF, CR increases levels of this protective factor in the dentate gyrus of the hippocampus, an important brain structure for learning and memory (Lee et al. 2001). Beta amyloid levels, on the other hand, have been shown to be reduced by CR in transgenic mouse models of AD (Halagappa et al. 2007), even when the CR is initiated in middle age (Mouton et al. 2009).

Table 10.2 Effects of CR observed in the brain

Positive age-related changes
Moroi-Fetters et al. (1989): Suppresses loss of dendritic spines
Gould et al. (1995): Improves adrenergic function cerebellum
Dubey et al. (1996): Reduces oxidative damage in striatum and hippocampus
Guo and Mattson (2000): Increases stress proteins (HSP-70, GRP-78)
Eckles-Smith et al. (2000): Prevents deficits in LTP and NMDA receptor decline
Magnusson (2001): Improves NMDA receptor function
Lee et al. (2001): Increases BDNF & neural stem cell expression in DG
Rotta et al. (2003): Decreases sensitivity to glutamate stimulation
Poon et al. (2006): Sustains proteins necessary for mitochondrial function
Shi et al. (2007): Prevents decline in NMDA and glutamate receptors
Adams et al. (2008): Stabilizes protein levels in CA3 across lifespan
Minor et al. (2009): Increases neuropeptide Y in arcuate nucleus
Mouton et al. (2009): Decreases amyloid plaque formation APP/PS1 mouse
No age-related changes
Newton et al. (2005): Age increases BDNF but no effect of CR in CA1, CA3 or DG

Recently, the hypothesis of neurohormesis has been proposed to explain why CR would benefit brain aging and function (Mattson and Cheng 2006). According to this hypothesis, CR induces a mild stress in organisms at a molecular level that generates a protective state by inducing the upregulation of protective proteins and pathways. In addition to CR, select dietary and environmental interventions may also induce neurohormesis; resveratrol, a polyphenol isolated from berries, as well as whole berry supplementation (Joseph et al. 2005; Pearson et al. 2008) have been shown to be beneficial to brain function and may be subsumed under this hypothesis, as has environmental enrichment (Frick and Fernandez 2003; Mattson et al. 2001).

One issue that has not been entirely explained is why the brain under CR would not be damaged by the chronic but moderately increased levels of circulating corticosterone (CORT). A transient elevation in CORT can have a beneficial effect on synaptic function and neuroplasticity, but chronic elevation has been implicated in cellular damage and mild cognitive impairment in the aging hippocampus (Arbel et al. 1994). Patel and Finch (2002) have suggested that CR may generate relatively mild levels of CORT and that chronically higher circulating levels of CORT would be required to damage the hippocampus. However, the effects of chronic, moderately elevated levels of CORT induced by CR have not been systematically investigated in the context of the aging brain and its impact on learning and memory and should be more thoroughly studied.

10.3 Assessment of Age-Related Deficits in Learning and Memory in Rodents

Care must be taken in the implementation and interpretation of experiments designed to assess the effects of CR on learning and memory in aged rats and mice. Among the caveats that the experimenter may face are (a) changes in sensation and perception with age in animals that might differentiate aged AL from CR animals, e.g., retinal degeneration, loss of hearing, changes in taste sensitivity (Ingram et al. 1994), (b) the possibility that the effects are a result of development and not aging, (c) the development of pathology or disease states that may differentially interfere with brain function in AL versus CR animals, e.g., cancer, physical immobility, and (d) differences in the level of motivation between “hungry” CR animals and their AL counterparts as well as how they experience satiety or fullness upon food consumption.

These issues are recognized by most gerontologists that study aging in rodent models, but not all CR studies are conducted by experts in these fields. For their part, some aging journals are beginning to encourage the use of three separate age groups to be compared in cross-sectional studies (i.e., representative young, middle-aged and aged groups are required) with the additional requirement that the aged group be at or beyond the mean or median lifespan for the specific strain under study. Some journals also ask that the specific pathogen status of the colony be reported as even the existence of various pathogens may affect experimental outcomes, including behavior.

The most crucial factor in assessing age-related deficits seems to be the complexity of the task (Ingram et al. 1994). Rats and mice seem most suited for learning mazes and are able to navigate in their environment utilizing spatial cues. Thus, it is not surprising that many studies of CR in aging rats and mice utilize complex mazes such as the Morris water maze, the 14-unit T-maze, and the radial arm maze. Simpler tasks may be employed such as fear conditioning, T-maze alternation, delayed matching to sample or nonmatching to sample but the utilization of time delays to increase the mnemonic load are generally required to observe age differences (Ingram et al. 1994).

Using food as a motivator for behavior studies is a potentially confounding variable as the CR animal is not only hungrier but also experiences satiety differently than the AL fed animal (Ingram 1994). The addition of age as a variable exacerbates this confound. For instance, aged monkeys have recently been observed to be less motivated to work for food (Mattison et al. 2005). The issue of hunger and motivation in relation to aging, CR and learning and memory assessment will require further systematic study to determine its level of involvement in behavioral outcomes. Furthermore CR animals are smaller, more agile, and may respond differently in response to the motivation utilized. For example, CR animals may fare better than their AL counterparts in tasks that assess latency to achieve a goal simply because they are leaner and faster. In the Morris water maze in particular, latency to escape to a submerged platform utilizing spatial cues may not be a justifiable comparison due to size and speed differences between CR and AL animals (Carter et al. 2009).

Interpretation of aging studies with rodents is often complicated by sensory issues such as visual impairments caused by retinal degeneration in certain mouse strains or cataracts that are characteristic of albino rats, e.g., F344. The investigator may choose to either utilize a task that does not require visual cues, such as the 14-unit T-maze (Ingram 1985), or demonstrate that vision is not a confounding issue as by reporting time to swim to a visible platform in the Morris water maze (Lindner 1997). Likewise, when using simpler tasks such as tone-cued shock avoidance to assess age-related impairments, the investigator must make certain that hearing is not impaired. The commonly-used C57BL/6 mouse experiences hearing loss at high frequencies beginning at 5–6 months of age so tone-cued studies should not be employed to assess age or CR differences (Ingram et al. 1994).

The development of new technologies including software and image analysis systems has made it easier for investigators to utilize a wide range of dependent measures, thus encouraging investigators to choose to report only outcome variables that support their hypothesis. These technologies when used with behavior tasks that generate data on multiple variables may engender an unwanted situation where investigators can mine for results a posteriori to report significant differences even if they do not reflect results generated from a priori hypothesis testing. Recently Maei et al. (2009) assessed the most sensitive measure of probe trial performance using a Monte Carlo simulation including probe trial performance of 1,600 mice that served as controls in Morris water maze studies and found that the Gallagher measure (mean proximity to the platform) was the most sensitive measure.

10.4 CR Learning and Memory Studies with Aged Populations

In this section the results of 22 studies of the effects of long-term CR on learning and memory performance in aged rats (n=15) and mice (n=8) are reviewed and summarized separately by species. For the most part, the age at assessment of performance is at or beyond the mean or median lifespan in the rat or mouse strain reported. Increased funding from the National Institute on Aging, including the availability of aging rodent colonies of rats (F344, BN and F344 × BN F1) and mice (C57BL/6) on AL and CR diets have bolstered support for research in this area resulting in an increase in the number of published studies over the last decade.

10.4.1 Rats

While compiling the published studies that utilized senescent or pre-senescent rats, it became apparent that strain specificity is an important determinant in behavioral outcomes of CR studies. Table 10.3 represents all studies of cognition using some form of CR on aged rats. The strains are ordered by the ratio of the number of studies that reported positive effects of CR over the total number of reported studies. Looking at this table it seems evident that learning and memory performance is enhanced in the outbred (SD/CD Cobbs, W) or the hybrid (F1) strains. Summaries of these studies are provided below and the reader will note that controversy remains regarding whether the improved performance is due to agility and speed or genuine enhancement of cognition.

Table 10.3 Effectiveness of CR on learning and memory in rats

	BN	F344	W	SD/CD-Cobbs	F1
Positive studies	0	1	1	2	3
Total studies	1	4	3	3	4
CR %	40	40	EOD[a] and 50[b,c]	EOD[d] or 20[e,f]	40

[a]Goodrick (1984), [b]Bond et al. (1989), [c]Yanai et al. (2004), [d]Beatty et al. (1987), [e]Pitsikas et al. (1990), [f]Pitsikas and Algieri (1992).

With respect to the ratio of positive studies with CR, the BN rat displays the least improvement, although this likely reflects the paucity of data from this strain. Markowska and Savonenko (2002) observed no difference in performance in the Morris water maze in the aged BN rat on CR compared to AL counterparts. Similarly, CR does not seem to be strikingly beneficial to the relatively lean albino

F344 rat. In the Morris water maze Markowska (1999) failed to find positive effects of CR in a cross sectional study of 6, 12, 18 and 24 month-old AL and 40% CR rats. At 18 and 24 months of age both AL and CR rats experienced decline in spatial place learning. Likewise Stewart et al (1989) found no benefit to old F344 rats after 40% CR in either the Morris water maze or in the 8 arm radial maze. Our laboratory (unpublished data) has also observed that both AL and CR F344 perform equivalently in a shock-motivated (0.8 mA) 14-unit T-maze at old age. Only one study, using a complex motor learning task in 22 month-old F344 rats found the CR animals were faster to learn to move across a series of pegs of regular or irregular patterns to obtain water (Gould et al. 1995). These results may again reflect the fact that the CR rat is much smaller and more agile.

Regarding Wistar (W) rats, neither Bond et al. (1989), nor Yanai et al. (2004) found significant improvements to cognition with CR. Bond et al. (1989) assessed radial arm maze performance in male W rats restricted to 50% of AL consumption. Compared to a young adult group (5 month old at start of testing) both the CR (28–30 month old at start of testing) and the AL group (26–28 month old at the start of testing) performed poorly in the radial maze task as assessed by correct arm entries across 15 test days. It should be noted that most studies do not restrict animals at this level, usually restriction is 30–40%. Thus, the relatively severe 50% restriction may be the reason for the negative results in this study. Yanai et al. (2004) assessed learning performance across the lifespan in AL and CR W rats. In this study, learning was impaired in all tasks in the CR rats compared to the AL rats across the lifespan but the CR rats did live longer. Body weights in the CR group were maintained at a level 65% less than AL for most of their lifespan (CR=280 g across the lifespan versus AL=800 g) which is not observed in most CR studies so like the previous study severe CR may be responsible for the negative results. This observation is reinforced by the finding that when CR rats were administered a peripheral injection of 500 mg/kg glucose 30 min prior to training, their learning deficit was eliminated and they performed equal to AL counterparts. The one positive result published on the W strain is also the oldest (Goodrick 1984) and observed that W rats fed EOD were superior in the 14-unit T-maze at 30 months of age compared to 24 month-old AL and equivalent to 6 month-old rats when food was used as a reward. In that study the rats completed three trials in the maze every other day for 10 days (trials=30) on days when the EOD rats were not fed.

In the outbred Sprague-Dawley (SD) rat Beatty et al. (1987) observed that an every other day feeding regimen from 3–21 months of age for CR rats was not beneficial to performance in an 8 arm radial maze compared to their age-matched counterparts when delays were imposed between the fourth and fifth trials (0, 1 and 5 h time intervals). In contrast, Pitsikas et al. (1990) found Morris water maze performance in male CD-COBS (a substrain of the SD) was improved by a hypocaloric (HYPO) diet that resulted in an average 20% CR. Most importantly, all rats showed improved learning curves in the water maze evidenced by decreases in latencies and distance swum to the platform. Similarly, in a separate report Pitsikas and Algeri (1992) reported that 3, 11, and 25 month old HYPO diet rats learned both spatial and non-spatial versions of the 8 arm radial water maze better than the AL rats.

The hybrid F344 × BN F1 (F1) rat seems to fare better cognitively on CR in later age than do either the F344 or BN inbred strains (see Table 10.3). Markowska and Savonenko (2002) assessed performance of male F1 rats at 9, 18 and 30 months of age. The CR F1 rats did not experience decline in place discrimination performance at 30 months of age while AL rats did. Fitting et al (2008) assessed spatial learning performance of male F1 rats in the Morris water maze in 6 month AL, 36 month AL and 36 month CR groups with 20 trials across 3 days of testing. Age and CR effects for escape latencies were observed with 36 month CR rats exhibiting significantly shorter latencies compared to the 36 month old AL rats on days 2 and 3. Interestingly, the authors indicate that CR did not assert a learning effect however the time spent in the target quadrant during the probe trial for the 36 month old CR rats was above chance while that of the 36 month old AL rats was not, indicating a potential benefit of CR. Adams et al. (2008) reported both AL and CR rats experienced cognitive decline in Morris water maze performance but the effects declined across all age groups in the AL rats whereas CR rats experienced decline between young and middle age but not between middle age and the old rats. Most recently, Carter et al. (2009) tested AL and CR male F1 rats from the NIA colonies between 8 and 38 months of age on two tasks: object recognition and the Morris water maze. The CR rats were universally poorer than AL rats on object recognition, and the authors state this is likely due to inability to focus caused by an increase in physical activity in CR rats. In the Morris water maze the age-related impairment was attenuated by CR for measures of latency to reach platform and time spent in proximity to platform but no differences were observed in the distance traveled to reach the platform, which the authors argue is a more sensitive measure of learning.

10.4.2 Mice

The oldest study to examine learning in aged CR and AL mice used C3B10RF mice (Ingram et al. 1987). Aged (31–35 months of age) female C3B10RF mice on 40% CR made significantly fewer errors in a shock-motivated version of the 14-unit T-maze than did their age-matched control (AL) mice.

The well-characterized C57BL/6NIA (B6) mouse is a common subject for aging and CR studies due to its availability from colonies available from the National Institute on Aging. Means et al (1993) assessed behavior of B6 mice in a battery of psychomotor, learning and memory tasks that were maintained on either a high calorie (HIC) or low calorie (HYPO) diet from middle age (∼14 month). In a Y maze the percentage of spontaneous alternation (an evolutionarily-preserved protective response in mice and rats) was significantly higher in the HYPO group than either a 12 month old or 22 month old HIC group. No differences were observed in the plus maze, a measure of emotionality. In the Morris water maze (3 trials per day for 6 days) escape latencies revealed learning in each group but no differences in latency to escape to the hidden platform (reported as log latency).

In a study by Bellush et al. (1996) no differences were found in two experiments assessing age-related differences in spatial water maze learning in B6 mice. In these experiments 2 month AL and 22 month AL and 40% CR mice were trained in a cued learning task to swim to the platform. The same year, Dubey et al. (1996) found B6 mice on 40% CR do not exhibit the declines in locomotor activity or rotarod performance associated with age in AL controls. They also observed an attenuated loss in learning in an active avoidance task where 16 month old CR mice were better than the AL group. However, the CR and AL groups showed a similar decrement to learning from 16 to 22 months.

Magnusson (1997, 2001) has reported beneficial effects of CR in a series of spatial water maze studies in the aging B6 mouse and observed that the effect of CR was correlated with improved glutamatergic function (specifically in the NDMA receptor) in brain areas important to learning and memory (Shiarella and Magnusson 1995; Magnusson 1998). The initial report compared the performance of 3 month old AL and 10 and 26 month old AL and CR mice. The aged AL mice performed poorly compared to all other groups in water maze performance (by the Gallagher measure) while the aged CR group performed better and were significantly worse than only the 3 month old group. The latter report (Magnusson 2001) assessed 3, 15 and 26–27 month old AL and CR B6 mice finding behavioral results similar to the initial study in the spatial water maze. Additionally, learning in place discrimination tasks and a probe trial (average proximity to the removed platform) was the poorest in the oldest AL group compared to the other groups while the old CR mice performed at a level not significantly different from younger groups.

CR also appears to benefit the lifespan of the long lived Ames dwarf mouse, even though this mouse experiences a primary pituitary deficiency which renders it deficient in growth hormone, prolactin, and thyroid stimulated hormone (Mattison et al. 2000). Male and female mice 18–21 month of age and fed 30% less than their AL counterparts were better at retaining memory, i.e., had longer latencies to enter a darkened chamber, in a shock-motivated inhibitory avoidance task. This improved inhibitory performance occurred even though the CR mice were more active than AL mice as assessed by locomotion in the home cage.

The only published report on transgenic mice used the 3xTgAD mouse model for Alzheimer's disease, which is on a B6 background (Halagappa et al. 2007). The researchers employed males and females of 3xTgAD and B6 controls fed either AL, 40% CR or EOD and aged either 10 or 17 months. At 10 months of age the genotypes were equivalent in Morris water maze performance, but at 17 months of age the AL-fed 3xTgAD mice were significantly worse than B6 controls. Both CR and EOD improved latency and path length measures in the aged 3xTgAD mice and brought their performance to the level of B6 controls.

10.5 Conclusion

The value of assessing the effects of long-term CR on learning and memory in animal models goes beyond its potential for delaying age-related brain dysfunction. CR could also be used as a positive control against which to compare the effects of other dietary interventions and exercise that may be more readily employed in human populations like fruits and vegetables or their individual components like resveratrol. However, after focusing this review on the effects to learning and memory in rats and mice on long-term CR at advanced ages (at or beyond the mean or median lifespan for the strain) compared to AL counterparts, we conclude that positive effects of long-term CR do not extend to all strains of rats or mice studied. Level of CR and body composition and the methods used in behavior tasks are just some of the variables that appear to impact the outcomes of these studies.

The effects of CR are known to be modulated by the degree of CR. Regarding lifespan, increasing the level of CR can increase the extension in lifespan up to a point after which mortality is increased as a result of starvation. CR's effects on the brain, then, could logically be expected to follow a similar pattern where some levels of CR would be beneficial but would turn detrimental if increased beyond a critical level. Indeed, the studies reviewed in this paper suggest this effect can occur in rodents. For example, restriction to 50% of AL diet appears to be detrimental to learning and memory in the two studies in the W rat (Bond et al. 1989; Yanai et al. 2004). In particular, given the brain's heavy reliance on glucose as a fuel, these rats may be underperforming as a result of hypoglycemia demonstrated by the improved performance following glucose administration (Yanai et al. 2004). Furthermore, a given level of CR may also affect unique strains differently depending on their body composition. Long-term 40% CR on brain function in the leaner inbred strains, i.e., F344, BN, appears to be more detrimental at later ages than in the heavier hybrid F1 or the outbred strains, SD and W.

The assessment of age effects in learning and memory requires that the tasks be sufficiently complex to elicit potentially subtle differences and the methodology employed while administering behavioral tasks is critical to discriminating differences. For example, the Morris water maze is the most common task used to assess age differences affected by CR and it appears that the actual protocol utilized may also affect the outcomes. For example, Markowska (1999) and Markowska and Savonenko (2002) utilized acquisition training coupled with multiple probe trials in determining effects of CR in rat F1 hybrids but not in the F344 or BN rat strains. Magnusson (1997, 2001) utilized a standard Morris water maze paradigm with 6 days of 3 trials and a probe trial at the end of training every other day starting on the second day to establish CR differences in the aged B6 mouse. Fitting et al. (2008) provided a 60 s probe trial by removing the platform on a third day following acquisition training in a place discrimination and observed minimal improvement in the CR F1 rats. This level of training and failure to employ a probe trial daily may be insufficient for aged, caged and un-enriched rats and may partly explain their negative results. Moreover, the Markowska and Magnusson studies utilized a collapsible Atlantis-type platform for the probe trial, enabling the mouse to climb on the platform once the probe trial ended. This method likely prevents confusion in the animals and may have contributed to the positive results in these studies. Magnusson also utilized the Gallagher method for assessing performance in probe trials, which has been suggested to be the best method for gauging success in the spatial version of this task (Maei et al. 2009).

One variable in CR behavior studies that is likely to become a critical issue in the near future is the use of transgenic models. This will further complicate the interpretation of findings as numerous transgenic strains with mixed backgrounds are introduced in order to ascertain neural and signal transduction pathways involved in learning and memory and to determine important mechanisms involved in CR effects. Investigators will need to recognize that the introduction of a human transgene into a mouse of a B6 background will most likely result in a range of behaviors quite different from the same transgene in an ICR or 129 background.

Clearly more studies need to be done with both rats and mice to better elucidate the effects of CR on brain aging. Specifically studies need to focus on the level and length of CR and the methods and tasks employed in behavioral assessment. It may be of practical interest to begin incorporating comparisons of CR with dietary supplements, exercise or even a high fat diet as additional points of interest. Funding agencies should be encouraged to support such preclinical investigations as a high priority due to the current and anticipated increases in numbers of aged individuals in developed nations throughout the world.

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Chapter 11
The Aging Liver and the Effects of Long Term Caloric Restriction

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11.1 Introduction

In the past it was proposed that the liver does not undergo significant aging changes because of its large functional reserve, regenerative capacity and dual blood supply (Popper 1986; Schmucker 1998). However, age-related changes in hepatic function are substantial and have potential impact on systemic exposure to xenobiotics (neurotoxins, carcinogens, pharmaceutics) and endogenous substrates associated with disease (lipoproteins, advanced glycation endproducts, immune complexes) (Table 11.1). Accordingly, age-related changes in the liver have implications for many diseases of aging and the aging process itself. Rather than the liver just being considered as a convenient source of tissue for general aging studies, the effects of aging on the liver per se have now been emphasized and considered an important focus for aging research (Boyer 2001; Geller and Zenick 2005; Schmucker 2005; Serste and Bourgeois 2006). The liver also has a pivotal role in coordinating metabolism in response to nutrition therefore many of the benefits of caloric restriction are likely to be mediated, at least in part, by the direct effects of diet on hepatic function. Indeed in 1985, Hans Popper proposed the concept ("thesis 1") that "The liver exerts several effects on the aging of the whole organism. This may offer an opportunity to delay or minimize senescence of the organism by modulation of hepatic activity for instance by diets or even drugs" (Popper 1986).

11.2 Hepatic Mass

One of the earliest descriptions of the aging liver was "brown atrophy", which is the reduction in liver mass associated with deposition of the aging pigment, lipofuscin (Popper 1986; Zeeh and Platt 1990; Porta 1991; Le Couteur and McLean 1998). The

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Table 11.1 Influence of old age on some hepatic metabolic and detoxification functions that are likely to impact on systemic disease and aging

Substrate	Age-related change decline in various measures of hepatic (%)	Citation
Xenobiotics (phase I and phase II)	∼20–60	(Le Couteur and McLean 1998; Butler and Begg 2008)
Chylomicron remnants and post-prandial lipoproteins	∼50	(Krasinski et al. 1990; Cassader et al. 1996)
Low density lipoproteins	15	(Ericsson et al. 1991)
Neurotoxins (MPTP)	75	(Yang et al. 2002)

Table 11.2 Some studies on the effect of old age on liver mass

Species	Age-related reduction in hepatic size as a fraction of body weight (% reduction compared to young)[a]	Citation
Humans	36	(Calloway et al. 1965)
	35	(Wilkinson 1997)
	17	(Bach et al. 1981)
	25	(Schnegg and Lauterburg 1986)
	29	(Wynne et al. 1989b)
Rats	30	(Varga and Fischer 1978)
	18	(Wiener and Rabinovici 1961)
	29	(Schmucker 1978)
	27	(Le Couteur et al. 1995)
	21	(Uchida et al. 1991)
	4	(Tuchweber et al. 1991)
	41	(Dawson et al. 2000)
Mice	2	(James et al. 1998)

[a] Old age was usually 24–36 months in the mouse and rat studies and at least 65 years in the human studies.

reduction in liver size as a fraction of body weight is usually in the order of 25–35%, although in some studies no major changes have been detected (Table 11.2) (Van Bezooijen 1984).

There is a parallel reduction in the number of hepatocytes (Tauchi and Sato 1975; 1978) that is likely to be secondary to the age-related increase in apoptosis of hepatocytes (James et al. 1998; Ando et al. 2002). Conversely, hepatocytes derived from long living Ames dwarf mice are even more likely to undergo apoptosis when exposed to oxidative stress in vitro (Kennedy et al. 2003) suggesting that increased hepatocyte apoptosis might be useful in delaying aging. Caloric restriction has usually been associated with a reduction in liver size even more marked

Table 11.3 Some studies of the effect of caloric restriction on liver mass in old age (expressed as percentage of body weight; AL ad libitum fed; CR caloric restricted)

Species/strain	Young AL (%)	Young CR (%)	Old AL (%)	Old CR (%)	Citation
Rat Wistar			3.04±0.07	2.95±0.08	(Valle et al. 2008)
Rat F344	3.0±0.2	2.4±0.2	3.3±0.1	2.4±0.1	(Jamieson et al. 2007)
Rat F344			~3.1	~2.8	(Greenberg and Boozer 2000)
Rat F344	4.1±0.3		2.72±0.04	2.64±0.10	(Leakey et al. 1989b)
Mouse B6C3F1	4.2±0.02	4.0±0.001	4.1±0.02	4.3±0.03	(James et al. 1998)
Rat Sprague Dawley	2.7±0.1	3.1±0.1	2.6±0.1	3.7±0.2	(Tuchweber et al. 1991)
Rat FBNF1	~2.9	~2.5	~2.9	~2.6	(Bevilacqua et al. 2005)
Rat F344			3.09	2.80	(Greenberg and Boozer 2000)
Rat F344			2.9±0.1	2.6±0.1	(Chou et al. 1995)

than that seen in age-matched controls (Table 11.3). Conclusions about the effects of caloric restriction on hepatocyte apoptosis are varied with some showing that apoptosis is enhanced at least in pre-neoplastic liver cells (Grasl-Kraupp et al. 1994; Muskhelishvili et al. 1996) while others have shown a reduction in stress-induced apoptosis (Cohen et al. 2004; Pearson et al. 2008). In passing it should be noted that aging in humans is now a major risk factor for non alcoholic fatty liver disease (NASH), which is becoming more prevalent presumably as a result of the obesity epidemic (Clark 2006). Therefore in the future, old age in humans may no longer be associated with brown atrophy of the liver, but in fact the opposite.

11.3 Hepatic Blood Flow

Most studies in humans and animals have shown that total hepatic blood flow is reduced in the order of 30–50%, which equals or exceeds the degree of age-related reduction in liver mass (Wynne et al. 1989b; 1990b; Le Couteur and McLean 1998; Schmucker 1998; 2001; Schmucker 2005). However, the haemodynamic responsiveness of the perfused rat liver does not change (Le Couteur et al. 1992) nor is there any increase in portal pressure in old age (Le Couteur and McLean 1998). Liver perfusion, which is the flow per mass of liver, is also reduced in old age but to a lesser extent than total blood flow. Using the clearance of colloidal albumin, liver perfusion in rats was reduced about 15% by age 36 months (Brouwer et al. 1985). Recently Ito et al. (2007) used high resolution in vivo microscopy to investigate aging in mouse liver. There was a 14% reduction in the numbers of perfused sinusoids between 1 and 27 months of age and a 35% reduction in sinusoidal blood flow. This was associated with a marked increase in the perisinusoidal expression of ICAM-1 and a consequential increase in leukocyte adhesion and sinusoidal plugging (Ito et al. 2007). Another study reported no major aging changes in sinusoidal perfusion, leukocyte adhesion or sinusoidal diameter. Even so, in this study there was a reduction in sinusoidal flow of about 30% between 3 and 24 months (Vollmar et al. 2002). As yet, there have been few studies, if any, that have reported the effect of caloric restriction on hepatic perfusion and blood flow. In other tissues, caloric restriction increased blood flow and perfusion in the brain (Lynch et al. 1999) and decreased blood flow in fat tissue (Crandall et al. 1984).

11.4 Hepatocyte Morphology

Hepatocytes have been inconsistently reported to increase in size with aging in rodents and humans while most reports show increased numbers of polyploid and binucleate cells (Schmucker 1976; 1978; Tauchi and Sato 1978; Engelmann et al. 1981; Popper 1986; Schmucker 1998). Hepatocyte mitochondria become larger and fewer in number, sometimes forming mega-mitochondria (Tauchi and Sato 1978; Sastre et al. 1996). There is a marked increase in the dense components of the cytoplasm consisting of lysosomes and lipofuscin deposits (Porta 1991; Schmucker 1998) and this feature is preserved in isolated hepatocytes (Van Bezooijen et al. 1974). Various other age-related lesions and pathologies have been reported in rodent livers including biliary dilatation and hyperplasia, focal areas of inflammation, fatty change, hepatomas and myelodysplastic infiltration but tend to be species- and strain-specific (Van Bezooijen 1984; Popper 1986; Lipman et al. 1999a). The loss of mitochondrial number was exacerbated by a high fat diet (Baur et al. 2006), while acute severe caloric restriction (one third normal food intake) in rats was associated with a decrease in hepatocyte size and glycogen content, and an increase in mitochondria number (Herdson et al. 1964).

The detailed effects of long term caloric restriction on organ morphology in rodents were reported recently (Lipman et al. 1999b, a). In B6C3F1 mice, there was an age-related increase in vacuolated hepatocytes, which was reduced by caloric restriction. Vacuolated hepatocytes were a key characteristic of aging in these mice. The presence of lymphocytes in the liver was also a feature of aging that was reversed by caloric restriction and presumably reflects the pro-inflammatory nature of old age (Lipman et al. 1999b). In a similar study of three strains of rat, aging was associated with bile duct hyperplasia and the presence of lymphoid nodules. As well as liver tumors in all strains, large granular cell leukemia was very common in old F344 rats. All these pathological changes were delayed by caloric restriction (Lipman et al. 1999a).

11.5 Sinusoidal Cells

Old age is associated with morphological changes in the hepatic sinusoidal endothelium and space of Disse from intact livers of many species including humans (Le Couteur et al. 2001; Cogger et al. 2003; McLean et al. 2003; Hilmer et al. 2005; Warren et al. 2005; Ito et al. 2007; Jamieson et al. 2007; Le Couteur et al. 2008; Stacchiotti et al. 2008). These changes have been called "pseudocapillarization" (Le Couteur et al. 2001). On electron microscopy there is an increase in endothelial thickness and a reduction in the porosity and number of fenestrations, which are pores in the endothelial cell that facilitate transport of particulate substrates (Le Couteur et al. 2008). These changes are associated with perisinusoidal basal lamina deposition in many old livers and some scattered collagen in the space of Disse (Lipman et al. 1999b, a; Le Couteur et al. 2008). The age-related changes in the ultrastructure of the sinusoidal endothelium are associated with altered expression of several antigens and stains used to identify pathology in the hepatic sinusoid (Le Couteur et al. 2008). In most studies the expression of von Willebrands factor is increased in old age, one study showed a reduction in caveolin-1 expression (Jamieson et al. 2007) and while another report showed increased ICAM-1 expression (Ito et al. 2007). There is upregulation of markers of extracellular matrix, such as collagen IV and Sirius red. These findings, together with the presence of collagen in the space of Disse on electron microscopy, are consistent with the development of patchy perisinusoidal fibrosis in old age. The age-related reduction in fenestration porosity is substantial (around 50%) and has several implications. In particular, the effect of the loss of fenestrations on the hepatic disposition of lipoproteins such as chylomicron remnants and consequent risk of systemic vascular disease has been reported (Le Couteur et al. 2002; Hilmer et al. 2005). The loss of fenestrations and altered diffusional properties of the aged sinusoidal endothelium and space of Disse also influence the hepatic clearance of medications, hence contributing to adverse drug reactions (Le Couteur et al. 2005). The effects of caloric restriction on pseudocapillarization have been investigated. In old caloric restricted rats, endothelial thickness was significantly less (190±7 nm vs 211±6 nm) and fenestration porosity was significantly greater (3.9±0.3% vs 2.4±0.1%) than in old ad libitum fed rats. In young rats, caloric restriction increased fenestration porosity above normal levels. Caloric restriction prevented the age-related decrease in liver caveolin-1 expression and content (Jamieson et al. 2007; Martini et al. 2007) and increase in perisinusoidal collagen IV staining (Jamieson et al. 2007).

The liver sinusoid also contains Kupffer cells (macrophages) and stellate cells (fat storing cells). There have been some studies of aging in these cell types, although none on the effect of caloric restriction. Two early studies of Kupffer cells in humans noted an increase in their numbers and activity in old age (Schaffner and Popper 1959; Findor et al. 1973). In rats, an increase in Kupffer cell numbers has been reported (Hilmer et al. 2007) whereas another study reported a reduction in their volume density (Martin et al. 1992). Kupffer cells from old rat livers have fewer pseudopodia and a reduction in the actin and myosin cytoskeleton (Sun et al. 1998) and an increased number of lysosomes (Knook and Sleyster

1976). The reported effects of age on Kupffer cell phagocytic activity are inconsistent. The uptake of colloidal carbon is either reduced (Videla et al. 2001) or unchanged (Vomel et al. 1981; De Leeuw et al. 1983; Yamano et al. 2000) whereas the uptake of denatured albumin has been reported to be reduced (Caperna and Garvey 1982; Heil et al. 1984; Brouwer et al. 1985). The uptake of large microspheres by Kupffer cells is either largely unchanged (De Leeuw et al. 1983; Vollmar et al. 2002), decreased (Sun et al. 1998) or increased (Hilmer et al. 2007; Ito et al. 2007). Kupffer cell uptake of other substrates such as mitochondria (Martin et al. 1994) and copper-ceruloplasmin (Vomel et al. 1984) is reduced in old age. The responsiveness of Kupffer cells to stimuli is diminished in old age as determined by carbon-induced oxygen consumption (Videla et al. 2001) and cadmium-induced phagocytosis (Yamano et al. 2000). Despite these inconsistencies, it is likely that the ability of the Kupffer cell to mount an effective immune response will be impaired given the established negative effects of aging on systemic immunity (Weng 2006). Furthermore, any increase in Kupffer cell number and basal activity is not unexpected (Hilmer et al. 2007) because aging is a pro-inflammatory state associated with increased systemic markers of inflammation (Johnson 2006).

Recently it has been found that stellate cells become swollen and engorged with fat in old age (Durham et al. 1990; Martin et al. 1992; Vollmar et al. 2002; Cogger et al. 2003; Grizzi et al. 2003; Warren et al. 2005; Ito et al. 2007). This is obvious on electron microscopy but also can be identified by a signet ring appearance on light microscopy (Cogger et al. 2003; Warren et al. 2005). The stellate cells in old age are not activated because they are engorged with fat, demonstrate increased vitamin A autofluorescence (Vollmar et al. 2002) and the expression of α-SMA and desmin, protein markers of stellate cell activation, is not increased (Cogger et al. 2003). Some of the stellate cells in rodents are so swollen that they protrude into the sinusoidal lumen it has been suggested that this will reduce sinusoidal blood flow (Ito et al. 2007).

11.6 Gene and Protein Expression

The effects of aging on gene and protein expression have become an increasing focus of research as a result of microarray and bioinformatic technologies (Raghothama et al. 2005). Patterns of altered gene expression with aging vary substantially between tissues and species, and results in the aging liver also vary between studies (Fu et al. 2006; Zahn et al. 2007; Lee et al. 2008a). Overall, it seems that there is much less age-related change in gene expression in the liver compared with other tissues (Zahn et al. 2007). Even so, expression of specific genes and proteins have been found to change in the liver. These have tended to show that there is decreased expression of genes involved with xenobiotic metabolism, mitochondria, apoptosis, and cell cycle/nucleic acid metabolism whereas there is increased expression of genes involved with inflammation. Caloric restriction reversed or prevents some but not certainly all of these aging changes in the liver (Cao et al. 2001; Fu et al. 2006; Pearson et al. 2008; Swindell 2008).

In livers from two rat strains, about 5% of 28,000 genes studied changed with old age. Apart from xenobiotic metabolism, the main gene groups different between young and old rats included those involved with metabolism of steroids and lipids, and the electron transfer chain (Lee et al. 2008a). In old Sprague Dawley rat livers, there was 1.7-fold or more increase in the expression of 17 and a 0.6-fold or less decrease expression of 30 genes out of the 1,000 expressed in the liver. These were mostly involved in energy metabolism including a reduction in genes involved in mitochondrial respiration and an increase of those involved with glucose and fatty acid oxidation (Tollet-Egnell et al. 2001). In a study of aging mouse livers, it was found that mRNA expression of only 20 out of 11,000 genes increased 1.7-fold or more with age. These were mainly pro-inflammatory genes (including lysozyme, biglycan, serum amyloid P component, cystatin B) and various other genes coding for stress response proteins and chaperones. Expression of another 26 genes decreased with age including those involved with cell cycle/DNA replication, IGF binding protein-1, xenobiotic metabolizing genes (N-sulfotransferase, 3 cytochrome P450s, glutathione-S-transferase omega-1) and apolipoprotein E (Cao et al. 2001). In another report of old mice livers, there was a statistically significant increase in the mRNA expression of 828 and decreased expression of 991 genes out of 12,000 tested (Fu et al. 2006). There has been at least one study in old humans, and in this study aging changes in gene expression were compared with those seen in rats. There was a threefold or greater change in expression was found in 582 genes in the old rat livers and 192 genes in old human livers. Antioxidant and the cytochrome P450 genes were influenced by aging in both rats and humans (Thomas et al. 2002). In addition there are age-related changes in liver microRNA (miRNA), which are short, non-coding RNA segments that suppress gene expression. With old age in mice there is an increase in the expression of 4 miRNAs. Approximately one quarter of the proteins down-regulated with age are targets of these up-regulated miRNAs, including proteins involved with cytoskeleton, proliferation, extracellular matrix, mRNA processing and ubiquination (Maes et al. 2008). However the total miRNA content did not change with age and the effect of age on miRNA is variable in other tissues and species.

Short term and long term caloric restriction reversed only some of the changes in gene expression that occurred in the aging mouse (Cao et al. 2001; Fu et al. 2006; Swindell 2008). For example, long term caloric restriction normalized the expression of 14 out of 20 genes that had increased expression in old age, and 13 of the 26 genes with decreased expression (Cao et al. 2001). In another study, there was little overlap between the effects of aging and caloric restriction on liver mRNA expression, in fact only 6 of 35 pairwise comparisons showed any overlap (Swindell 2008). Therefore, caloric restriction has a discrete effect on hepatic gene expression, which is not simply a reversal of age-related changes. On the other hand it is of particular interest that the SIRT1 agonist, resveratrol, closely mimics the effects of caloric restriction on hepatic gene expression in mice (Pearson et al. 2008; Swindell 2008). In the Pearson study, the gene expression patterns of 28 month old mice on caloric restriction or treated with resveratrol showed an 87 and 90% correlation with the expression patterns of mice 9 months younger (Pearson et al. 2008).

11.7 Hepatic Metabolism

The liver is responsible for coordinating much of the response to dietary manipulation. The hepatic metabolism of carbohydrates, protein and fat undergoes marked changes in response to starvation and feeding (Bauer et al. 2004). Insulin is the key hormone involved in regulating this response. In summary, old age is associated with hepatic insulin resistance, reduced protein and amino acid metabolism, reduced gluconeogenesis with increased glycolysis, and increased hepatic fat synthesis and storage. Caloric restriction has opposite effects as the liver utilizes protein for gluconeogenesis and liberates fat for systemic energy requirements.

11.7.1 Protein and Amino Acid Metabolism

Animal studies have shown an overall, but not consistent, reduction in total liver protein synthesis and degradation in old age (Van Bezooijen 1984; Ward and Richardson 1991; Salive et al. 1992; Fu and Nair 1998). Protein synthesis measured by valine uptake was found to decrease by 70% between 6 and 24 months in rats (Ward and Richardson 1991). Caloric restriction significantly increases the rate of protein synthesis by rodent livers (Ward and Richardson 1991). Albumin is the major protein produced by the liver and its blood level has usually been reported to decrease slightly in old age but remain within the normal range (Fu and Nair 1998; Thalacker-Mercer and Campbell 2008). In one human cohort study, albumin fell by 0.8 g/dL every decade of life and low levels correlated with mortality and frailty (Salive et al. 1992). Yet the fractional synthesis of albumin and its response to dietary feeding are not influenced by age in humans (Thalacker-Mercer and Campbell 2008). There is a reduction of the fractional synthesis rate of fibrinogen despite increased blood levels in older humans, indicating that there must also be an age-related reduction in fibrinogen clearance (Fu and Nair 1998).

The utilization of amino acids for gluconeogenesis is reduced in old age. This is secondary in part to the age-related reduction of protein production by skeletal muscle (Salive et al. 1992). Even so, hepatic alanine and aspartate transaminase decrease with old age (Hagopian et al. 2003a) and low circulating blood levels of alanine transaminase are associated with higher mortality in humans (Elinav et al. 2006). The hepatic uptake of amino acids (proline, serine, arginine) and urea production are also reduced in old rat livers. Alanine was taken up by young livers but in contrast was released by old livers where the hepatic content of alanine was increased fourfold (Jourdan et al. 2008). Caloric restriction is associated with increased utilization of protein for energy and there is increased expression and activity of enzymes involved in nitrogen metabolism. Caloric restriction increases the activity of hepatic transaminases in mice, presumably in order to increase gluconeogenesis (Hagopian et al. 2003a). Likewise, acute starvation also increases expression of hepatic transaminases and other urea cycle genes in mice reflecting the utilization of protein for energy production (Bauer et al. 2004). Long term caloric restriction in mice increased the expression of glutaminase and carbomyl phosphate synthetase 1, while reducing the expression of glutamine synthetase (Dhahbi et al. 2001). Given such changes in protein and amino acid metabolism with caloric restriction, it is surprising that many aspects of caloric restriction can be replicated by protein restriction, at least in rodents (Pamplona and Barja 2006). Protein restriction also generated longevity gains in drosophila independent of caloric intake (Lee et al. 2008b). Intriguingly, the changes in longevity caused by manipulating the protein content of the diet were inversely related to fecundity, which provides experimental verification of the disposable soma theory of aging proposed by Kirkwood (Kirkwood and Holliday 1979).

11.7.2 Carbohydrate and Glucose Metabolism

In general, the ability of the liver to supply glucose for systemic metabolism is diminished in old age. This is secondary to a combination of increased glycolysis and reduced gluconeogenesis. Old age is also associated with insulin resistance (Elahi and Muller 2000; Dhahbi et al. 2001) and this has been mechanistically correlated with abdominal fat that accumulates in old age (Gupta et al. 2000). There are increased fasting levels of insulin in old humans and rodents and there is probably a slight increase in blood glucose levels and glycosylated hemoglobin (HbA_{1c}) in humans (Elahi and Muller 2000). In the liver, insulin resistance is secondary to altered phosphorylation of the insulin receptor or early insulin signal transduction (Carvalho et al. 1996; Zhu et al. 2005). Age-related mitochondrial dysfunction also contributes to insulin resistance (Kim et al. 2008) and increasing circulating levels of insulin are, in part, secondary to altered hepatic clearance of insulin (Pacini et al. 1990). Caloric restriction preserves hepatic insulin sensitivity, particularly the ability of insulin to suppress hepatic glycogenolysis (Gupta et al. 2000; Dhahbi et al. 2001). Caloric restriction is associated with increased mRNA expression of the insulin receptor and increased phosphorylation of the insulin receptor following insulin stimulation (Zhu et al. 2004, 2005). The expression of insulin receptor and GLUT4 is not increased by caloric restriction (Masternak et al. 2005).

A consistent and important observation is the age-related reduction in the expression and activity of glucose-6-phosphatase, which is the final step in gluconeogenesis and glycogenolysis and has a pivotal role in regulating peripheral blood glucose levels (Tollet-Egnell et al. 2001; Hagopian et al. 2003a; Pearson et al. 2008; Swindell 2008). In old mice, there is increased expression of several key enzymes involved in glycolysis (phosphofructokinase-1, triosephosphate isomerase, enolase, pyruvate kinase) with an elevation of hepatic ketone bodies and reduction in hepatic lactate levels (Hagopian et al. 2003b). Microarray studies in the rat have also found reduced mRNA expression of gluconeogenic enzymes and increased expression of glycolytic enzymes (Tollet-Egnell et al. 2001) and there is reduced activity of enzymes involved in gluconeogenesis (glucose-6-phosphatase, fructose-1,6-bisphosphatase and transaminases) with an elevation of acetyl CoA

(Hagopian et al. 2003a; Swindell 2008). Lifelong caloric restriction in mice is associated with increased activity and expression of key hepatic enzymes involved with gluconeogenesis (pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase) while hepatic glucose and enzymes involved with glycolysis are reduced (Dhahbi et al. 2001; Hagopian et al. 2003b, a).

11.7.3 Lipid and Lipoprotein Metabolism

Liver fat and the levels of circulating cholesterol and triglycerides increase with old age. In one study total liver lipid content increased in old rats nearly threefold (Dawson et al. 2000) and in other studies in mice, there were increases in the liver content of cholesterol (Bose et al. 2005; Martin et al. 2008). As a result there is an increased prevalence of hepatosteatosis or non-alcoholic fatty liver disease in old age (Clark 2006). Hepatosteatosis is prevented in mice lacking either the peroxisome proliferator-activated receptor-alpha (PPARα) or L-fatty acid binding protein despite age-related obesity (Martin et al. 2008).

There are changes in expression of many proteins involved in hepatic lipid metabolism. There are increases in hepatic lipoprotein lipase, scavenger receptor B1 and apolipoprotein B but no change in HMG CoA reductase (Tollet-Egnell et al. 2001; Martin et al. 2008). PPARα participates in the control of fatty acid metabolism and beta oxidation through its effects on many genes involved in fatty acid metabolism. Therefore any age-related change in the expression of mRNA is likely to be a key factor in age-related changes in lipid metabolism. However, the expression of PPARα has been reported to be increased (Tollet-Egnell et al. 2001; Martin et al. 2008) or reduced in old age (Sanguino et al. 2005).

There is a reduction in the hepatic fractional catabolic rate of LDL in older humans by about 15% (Ericsson et al. 1991) and a reduction in the hepatic metabolism of chylomicron remnants by about 50% (Krasinski et al. 1990; Cassader et al. 1996). There are a variety of changes in enzymes involved in lipoprotein metabolism and disposition that may contribute to this impaired metabolism. For example, there is an increase in acyl-CoA:cholesterol transferase (Bose et al. 2005). Reports on the effect of old age on the LDL receptor vary although there is a consistent increase of LDL and LDL cholesterol (Bose et al. 2005; Galman et al. 2007) Although there is no change in total apolipoprotein E expression in old livers, the distribution is altered suggestive of impaired hepatocyte trafficking (Hilmer et al. 2004). Loss of fenestrations in the sinusoidal endothelium impairs the transfer of lipoproteins between blood and hepatocytes (Hilmer et al. 2005). Caloric restriction stimulates the use of fat for energy by increasing mitochondrial β-oxidation. Long term caloric restriction reduced total, LDL- and HDL-cholesterol towards levels seen in younger rats (Martini et al. 2007). Short term caloric restriction in rats enhanced the gene expression involved in fatty acid synthesis and those involved in mitochondrial β-oxidation, probably by increasing the expression of PPARα (Higami et al. 2006).

11.8 Hepatic Detoxification Pathways

Old age has usually been shown to be associated with reduced hepatic antioxidant, proteolytic and xenobiotic detoxification pathways, which potentially increases systemic exposure to toxins implicated in age-related disease. Caloric restriction has generally reversed such changes although the effects are variable.

11.8.1 Xenobiotic Metabolism

Xenobiotic metabolism occurs via two major pathways: the phase I pathway which is primarily the cytochrome P450 system, and the phase II pathway, which is conjugation. Both processes transform lipophilic substrates to hydrophilic substrates that can be excreted in bile or urine. Generally hepatic xenobiotic metabolism converts toxic substrates to their nontoxic metabolites however in some cases, substrates are activated to their active form. In old male rats there is a marked reduction in cytochrome P450 expression and activity, secondary to "feminization" of the male liver (Kitagawa et al. 1985; Kitani 1986; Birnbaum 1991). Cytochrome P450 content and most phase I pathways are normalized by caloric restriction, as are most of the phase II pathways. However the age-related decline in the hydroxysteroid metabolizing activity of UDP-glucuronyl transferase and sulfotransferase was not ameliorated, and some phase I pathways (microsomal testosterone 6β-hydroxylase, lauric acid 12-hydroxylase, 4-nitrophenol hydroxylase) were actually increased above young levels (Leakey et al. 1989a, b; Lee and Yu 1991).

In humans, the reduction in hepatic drug clearance has been ascribed primarily to age-related reductions in liver mass and blood flow rather than changes in enzyme content and activity (James 1985; Wynne et al. 1989a, b; Durnas et al. 1990; Schmucker et al. 1990; Wynne and James 1990). However, a recent detailed study of liver tissue from 226 humans found a 32% reduction in hepatic cytochrome P450 content after 70 years of age (Sotaneimi et al. 1997). In addition, impaired ability of albumin-bound drugs to cross the pseudocapillarized liver sinusoidal endothelial cell will contribute to altered drug metabolism (Le Couteur et al. 2005). It has been concluded that phase I drug metabolism is preferentially reduced in healthy old age while phase II metabolism is paradoxically preserved (Le Couteur and McLean 1998). However, in contrast to fit older people, phase II acetaminophen glucuronidation is reduced in the frail elderly (Wynne et al. 1990a). More recently a detailed re-analysis of hepatic metabolism has shown that old age is associated with a reduction in all metabolic pathways once free (non-protein bound) clearance is calculated (Butler and Begg 2008). Although conclusions regarding hepatic drug metabolism in aging humans have changed substantially over the last decades, current data suggest that most drug metabolism pathways are reduced by up to 50% in old age depending upon comorbidity and frailty, and that this is secondary to reduced hepatic blood flow and also probably hepatic mass and enzyme content.

11.8.2 Proteolysis and Autophagy

There are two major proteolytic systems, the ubiquitin-proteasomal and the lysosomal systems and these are present in all tissues. Autophagy refers to lysosomal proteolysis that is undertaken either to remove damaged proteins or to convert protein to energy in times of nutritional undersupply. An age-related decrease in autophagy occurs in most tissues including the liver and this presumably contributes to the increased levels of damaged proteins that accumulate within cells (Cuervo and Dice 1998; Vittorini et al. 1999; Cuervo 2008a, b). In particular, expression of the lysosomal receptor for chaperone mediated autophagy (LAMP2A) is reduced in the aged liver and transgenic upregulation of this receptor has been shown to delay age-related changes in hepatocyte and liver mitochondrial function and structure (Mizushima et al. 2008; Zhang and Cuervo 2008). The effect of caloric restriction on the age-related decline in lysosomal proteolysis in the liver was to partially restore its activity (Vittorini et al. 1999; Cavallini et al. 2001) and to maintain the responsiveness of hepatic autophagy to insulin and glucagon (Donati et al. 2008).

11.8.3 Endogenous Oxidant Metabolism

Old age is associated with increased oxidative stress and oxidative injury while endogenous antioxidant systems (such as superoxide dismutase, catalase, glutathione peroxidase, ascorbic acid, reduced glutathione) tend to decrease with age (Muller et al. 2007). Old age causes reduced expression of many hepatic antioxidant enzymes and increased evidence of oxidative stress in most studies, but such results are variable and probably influenced by strain and gender (Ji et al. 1990; Rikans et al. 1991; Amicarelli et al. 1997; Sanz et al. 1997; Martin et al. 2002; Muradian et al. 2002). In rat liver, there are reports of lower mRNA expression and activity of Cu/Zn superoxide dismutase, GSH reductase, catalase and glutathione peroxidase (Rikans et al. 1991; Sanz et al. 1997; Martin et al. 2002). The changes are likely to be significant, for example Cu/Zn superoxide dismutase activity was decreased to about 25–45% of that seen in young rats (Sanz et al. 1997; Kiray et al. 2004). Another study reported reduced activity of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in livers of old rats and also lower levels of ascorbate, α-tocopherol and glutathione associated with consequential increased levels of oxidative stress determined from lipoperoxides and protein carbonyls (Navarro and Boveris 2004; Senthil Kumaran et al. 2008). Reduced glutathione and the GSH:GSSG ratio in the liver decline with age (Sanz et al. 1997). As a consequence of the reduced antioxidant defenses, aging increases the susceptibility of hepatocytes to oxidative stress. There is more rapid loss of viability at lower concentrations of tert-butylhydroperoxide in hepatocytes isolated from old versus young rats (Hagen et al. 2000) with similar results obtained using hydrogen peroxide (Ikeyama et al. 2002; Li and Holbrook 2003). A decline in the ratio of coenzymes Q 10:9 and alpha-tocopherol in liver cell membranes was found in aged rat livers, and this change was attenuated by caloric restriction (de Cabo et al. 2004). In addition coenzyme Q-dependent NADPH dehydrogenases were also increased by caloric restriction and, as a result, liver cell membranes were more resistant to oxidative stress (de Cabo et al. 2004).

11.8.4 Endocytosis

Liver sinusoidal endothelial cells play a critical role in the removal of macromolecular waste products from the systemic circulation and are the most active endocytic cells in the body (Smedsrod et al. 1990; Smedsrod 2004). They clear many substrates of relevance to old age including connective tissue macromolecules, oxidized and acetylated low density lipoproteins, advanced glycation end products and immune complexes (Skogh et al. 1985; Smedsrod et al. 1997; Martin-Armas et al. 2006). In vivo microscopy was used to detect endocytosis of two fluorescent labeled scavenger receptor ligands, advanced glycation end product-modified albumin and formaldehyde-treated albumin in aged mice (Ito et al. 2007). There was suppressed endocytic function in old mice, especially in the pericentral area. It was concluded that such an age-related reduction in this activity increases extrahepatic deposition and deleterious effects of circulating waste macromolecules. The effect of caloric restriction on endocytosis has not been reported.

11.9 Hepatocyte Mitochondria

There are well established aging changes in the structure and number of liver mitochondria as well as increased free radical production and decreased ATP production and respiration. These changes parallel those found in other tissues (Hagen et al. 1997, 1999). In hepatocytes, there is a 30% reduction in mitochondrial potential (Sastre et al. 1996) and a 25% reduction in ATP measured using magnetic resonance spectroscopy (Le Couteur et al. 2001). The activity of many mitochondrial enzymes (eg mitochondrial nitric oxide synthase, Mn-superoxide dismutase, complex I, complex IV) is decreased in old age by 25–75% (Navarro and Boveris 2004). These dysfunctional mitochondria are a potent source of endogenous reactive oxygen species and contribute to age-related oxidative stress (Sastre et al. 2003; Lopez-Lluch et al. 2008).

There are aging changes in genes related to fatty acid oxidation and oxidative capacity and point mutations in mitochondrial (mt)DNA at specific regions that control replication of mtDNA (Ames et al. 1995; Sastre et al. 2000; Kujoth et al. 2005). The number of copies of mtDNA is reduced by about 50% (Barazzoni et al. 2000) and the age-associated 4,834 bp mtDNA deletion seen in other tissues also occurs in the aging liver (Cassano et al. 2004). Microarray studies of rat liver show additional age-related decline in expression of several nuclear genes involved in mitochondrial respiration (Tollet-Egnell et al. 2001).

The effects of caloric restriction on hepatic mitochondria have been extensively studied. The lactate:pyruvate ratio, a measure of cytoplasmic redox status and the hydroxybutyrate:acetoacetate, a measure of mitochondrial redox status did not change in the livers of aged mice, but are both increased by lifelong caloric restriction (Hagopian et al. 2003a). Caloric restriction increases the number of mitochondria in hepatocytes towards levels seen in young rats (Lopez-Lluch et al. 2006) and leads to less free radical production (Lopez-Torres et al. 2002; Lopez-Lluch et al. 2006) and less evidence of oxidative injury (Lambert et al. 2004; Lopez-Lluch et al. 2006). Despite lower oxygen consumption and free radical production, the mitochondria after caloric restriction maintain higher levels of ATP production (Lopez-Lluch et al. 2006). The effects of caloric restriction are mostly on complex I of the electron transfer chain (Pamplona and Barja 2006) and are mediated by increased activity of PPAR-1α (Lopez-Lluch et al. 2006). Caloric restriction also reversed the age-related accumulation of the 4,834 bp mtDNA deletion (Cassano et al. 2004) and oxidative damage to mtDNA (Lopez-Torres et al. 2002). It should be noted that as well as contributing to the aging process, impaired hepatic mitochondria are key factors in the pathogenesis of hepatosteatosis and hepatic insulin resistance, which are very common conditions in older people (Wei et al. 2008).

11.10 Regenerative Capacity and Transplantation

Hepatic regeneration following partial hepatectomy is complete but slower in old rats, taking up to 3–4 weeks rather than 1 week to fully reconstitute (Bucher et al. 1964; Beyer et al. 1991; Smanik et al. 1991). This slower regeneration is associated with decreased DNA synthesis and hepatocyte mitotic activity (Schapiro et al. 1982; Beyer et al. 1991; Bland et al. 1991; Chou et al. 1995; Shaddock et al. 1996). Recently it has been shown that the proliferative response in old age is reduced because of inhibition of c-myc mediated by C/EBPα (Iakova et al. 2003), reduced activation of extracellular signal-regulated kinase (ERK), reduced phosphorylation of the epidermal growth factor receptor (EGFR) and increased activation of p38 (Li and Holbrook 2003).

Livers transplanted from 1 year old rats were associated with less growth and lower albumin levels in recipient rats, compared with livers from 11 week old rats (Koh et al. 2006). In humans, transplantation of donor livers is associated with a 1% rate of primary graft failure when the donor is less than 55 years, compared with 10% with older donors (Serste and Bourgeois 2006). On the other hand, re-transplantation of livers in rats found that transplanted livers survived until maximum 52 months (compared with the maximum life of rat 39.9 months) and recipients had same survival whether given livers from 5 or 28 month donors. However, there was a higher incidence of fibrosis, pigment deposition and biliary proliferation in old transplanted livers (Sakai et al. 1997). Parabiosis experiments showed rejuvenation of old livers following union of young and old rats (Tauchi and Sato 1978) indicating that the re-transplantation studies might be a result of the rejuvenating milieu of the young recipients rather than an inherent quality of the old livers. Overall, it appears that old age impairs hepatic regeneration but transplantation of old livers is still feasible.

Caloric restriction accelerates liver regeneration in old age. Following partial hepatectomy in old rats, the percentage of liver regenerated after 24 h was 21% in control rats and this was improved to 29% with caloric restriction (Chou et al. 1995). Caloric restriction was associated with larger increases in DNA synthesis and hepatocytes in S phase following partial hepatectomy (Chou et al. 1995) and enhanced proliferative response to epidermal growth factor (EGF) (Ikeyama et al. 2003).

11.11 Mechanisms for the Hepatic Effects of Caloric Restriction

Although caloric restriction delays the aging phenotype and extends longevity, the data presented here show that caloric restriction can not be interpreted as simply reversing or slowing age-related changes in the liver. For example, caloric restriction does not normalize the effects of aging with respect to liver mass, apoptosis, endothelial fenestrations, xenobiotic metabolism and gene expression. Rather than being "anti-aging", caloric restriction, at least in the liver, can be considered as an intervention that ameliorates some of the deleterious phenotypic effects of old age. From an evolutionary perspective, it has been proposed that the beneficial effects of caloric restriction evolved as a positive switch to increase cellular resilience in times of famine (Holliday 2006). By contrast, in the past there has not been any evolutionary pressure to generate strategies to delay aging changes.

Recent studies have shown that the sirtuin pathway mediates many of the beneficial effects of caloric restriction and as such is the positive switch that responds to dietary deprivation (Cohen et al. 2004; Guarente and Picard 2005; Sinclair 2005; Haigis and Guarente 2006; Sinclair and Guarente 2006; Guarente 2008) (See Chapter 9). Sirtuins are one of the three classes of histone deacetylases, which are NAD+-dependent enzymes that deacetylase lysine groups found on histones. The sirtuins are named for their effect on histones and hence gene transcription (Silent Information Regulator) but also deacetylate lysine moieties on many other cellular proteins including p53, tubulin and PPARγ coactivator 1 (PGC-1α). Amongst the sirtuins, the SIR2 enzymes (consisting of SIR2 in yeast and SIRT1 in mammals) are most closely linked with longevity, caloric restriction and aging (Guarente and Picard 2005; Sinclair 2005; Haigis and Guarente 2006; Sinclair and Guarente 2006). Following long term caloric restriction in rats, there was increased expression of SIRT1 in all tissues including liver (Cohen et al. 2004). However, short term caloric restriction in mice showed reduced hepatic expression of SIRT1, while other tissues had the expected increase in SIRT1 expression (Chen et al. 2008). It was noted in this study that caloric restriction was associated with an increase in the NAD+/NADH ratio in muscle but a decreased ratio in liver, thereby potentially explaining the differential effects on SIRT1 expression. Resveratrol is an agonist of SIRT1 that recapitulates many of the effects of caloric restriction, and more potent agonists such as SRT1720 have recently been published (Howitz et al. 2003; Wood et al. 2004; Baur et al. 2006; Milne et al. 2007; Feige et al. 2008; Pearson et al. 2008). In the liver, resveratrol mimicked the transcriptional profile generated by caloric restriction (Pearson et al. 2008). In aged mice fed a high caloric diet, resveratrol prevented the development of fatty liver and its sequelae such as insulin resistance and hyperlipidemia (Baur et al. 2006).

11.12 Conclusions

Contrary to past assumptions, the liver does indeed undergo changes in structure and function in old age. Albeit, the changes in liver structure are subtle but the changes in function are often substantial, often in the order of 50% reductions in metabolic activity. Because of the central role of the liver in metabolism and detoxification, changes in liver function have potential implications for systemic aging and age-related diseases in particular cardiovascular disease. The effects of caloric restriction in the liver are beneficial but do not simply reverse or delay age-related changes, instead acting primarily through discrete cellular mechanisms such as the sirtuin pathway. Given the key role of the liver in responding to dietary perturbations, it is likely that many of the effects of caloric restriction are mediated by the liver.

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Chapter 12
Food Restriction, Hormones, Genes and Aging

Arthur V. Everitt, Holly M. Brown-Borg, David G. Le Couteur, and Andrzej Bartke

12.1 Introduction

Studies conducted over 40 years ago suggested that factors produced by the pituitary gland affected age-related physiological processes and disease development (Everitt and Duvall 1965; Everitt et al. 1968) and also longevity (Everitt et al. 1980). Pituitary hormones control multiple physiological functions that have been implicated in the regulation of aging including growth and body size, metabolism, reproduction and stress resistance. There is also a large literature showing that calorie restriction (CR) retards aging processes and prolongs life in rodents (McCay et al. 1935; Weindruch and Walford 1988a; Yu 1994; Masoro 2005; Piper and Bartke 2008). CR, by reducing the secretion of hormones from the pituitary and target glands, also affects the rate of aging and the development of many age-related diseases (Everitt and Burgess 1976; Everitt 2003). Hypophysectomy has been shown to retard aging and delay the development of certain pathologies in old age (Everitt 1976a). Specific anterior pituitary hormones may regulate aging processes and longevity; a central player under intense study is growth hormone. Interactions of growth hormone-related mutations and CR have revealed additional insight regarding mechanisms that control aging and longevity.

12.2 Calorie Restriction and Aging

In 1935 Clive McCay at Cornell University clearly showed that long-term CR begun at puberty in the rat extended the maximum lifespan. This is a robust effect confirmed in other research laboratories demonstrating that CR without malnutrition, when implemented after puberty, increased both median and maximum

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lifespans and inhibited the development of certain pathologies (Berg and Simms 1960; Weindruch and Walford 1988a; Yu 1994; Weindruch and Sohal 1997; Masoro 2005). CR plays a major role in delaying the onset of age-related diseases (Berg and Simms 1960; Everitt et al. 1982; Harman 1988; Meydani 2001; Stern et al. 2001; Polidori 2003). The life-extending effect of CR appears to be independent of the composition of the diet (Masoro 2002). The magnitude of the life extension depends on the age when CR is started, the degree of restriction and the animal strain (Weindruch and Walford 1982). Retarded aging in CR rats may be due to reduced secretion of pituitary hormones (Armario et al. 1987).

12.3 Hypophysectomy and Aging in the Rat

For many years CR has been termed a "functional hypophysectomy" because low levels of pituitary hormones are seen in CR animals (Mulinos and Pomerantz 1940; Campbell et al. 1977; Armario et al. 1987; Herlihy et al. 1990; Gautsch et al. 1998). The secretion of most hormones is greatly reduced by CR except corticosterone (Han et al. 2001). In the conventional male Wistar rat, hypophysectomy (HYP) reduces food intake, heart rate, hemoglobin, white cell count and creatinine excretion (Everitt 1976a), retards aging in tail tendon collagen fibers (Everitt et al. 1968), inhibits urinary protein excretion (Everitt and Duvall 1965), slows enlargement of the heart (Everitt 1976b), decreases aging of the gastrocnemius (Everitt et al. 1985) and soleus (Shorey et al. 1993) muscles and reduces the incidence of gross pathology at autopsy in old age (Everitt and Meites 1989).

12.4 Comparison of the Anti-aging Effects of Hypophysectomy and Calorie Restriction

In rats and mice long-term HYP, like CR, inhibits many age-related changes in functions of the immune system, metabolism, blood vessels, collagen, kidney, muscle, ovary, etc (Weindruch and Walford 1988b; Everitt and Meites 1989). It has been suggested that reduced aging in HYP rats may be due to their low food intake, and not their lack of pituitary hormones (Weindruch and Walford 1988b). This possibility was studied over a period of 20 years commencing in 1968 at the University of Sydney (Everitt 1971; Everitt et al. 1980). A direct comparison was made of the anti-aging effects of CR and HYP in conventional male Wistar rats consuming the same amount of food. HYP rats received weekly subcutaneous injections of 1 mg of cortisone acetate. CR rats were fed the same amount of food as HYP animals. HYP and CR rats were directly compared with intact controls (INTACT) starting at age 60 days.

Collagen fiber aging in rat tail tendon was measured by the breaking time of isolated fibers under a load of 2 g in 7 molar urea (Everitt and Wyndham 1982). The breaking times increased from about 1 min at age 60 days to about 80 min in

HYP, 120 min in CR and 225 min in INTACT controls at 800 days or 25 months (Fig. 12.1). Breaking times at 25 months were significantly different (*P*<0.01) from each other (Everitt and Wyndham 1982). Thus the physiological aging of collagen fibers was significantly reduced by CR and HYP, with HYP having a significantly greater anti-aging action than CR on the same food intake.

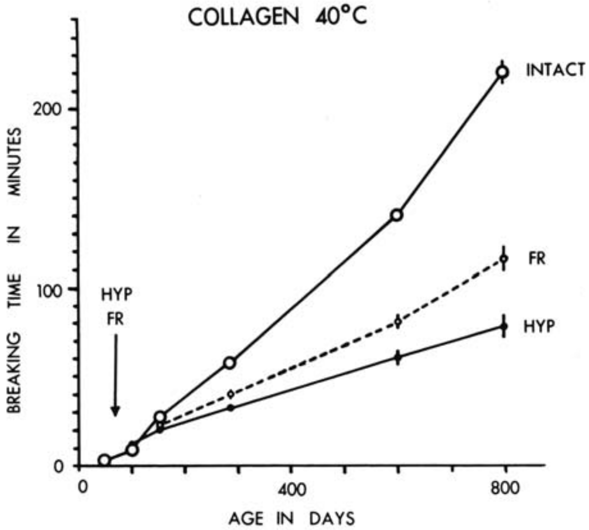

Fig. 12.1 The effect of hypophysectomy (HYP) and food restriction (FR) starting at age 70 days on the aging of rat tail tendon collagen fibers in intact control (INTACT) male Wistar rats, as measured by breaking time in minutes (mean ± SEM) of a 2 g loaded isolated fiber in 7 Molar urea at 40°C. HYP and FR rats consumed the same amount of food (Adapted from Everitt 1971)

Chronic glomerulonephrosis develops in the kidney of the aging male Wistar rat and can be followed by measuring the rise in urinary protein excretion reaching 50 mg/day at age 1,200 days. Both CR and HYP prevent the rise in proteinuria (Everitt et al. 1980). The glomerular basement membrane thickens with age and at 1,200 days measures 1,000 nm in INTACT rats, 550 in CR and 400 in HYP (Wyndham et al. 1987). Thus HYP has a greater anti-aging action on the glomerulus than CR (Fig. 12.2) in the male rat. In 1980 Johnson and Cutler reported that hypophysectomy inhibits various structural age-related changes in the glomerulus of the female rat.

Thoracic aorta wall thickness increased with age in all three groups. Following HYP or CR wall thickness declines and then rises with age but is significantly less than INTACT rats at all ages (Everitt et al. 1980). At three ages HYP rats had significantly lower aortic thickness than CR (Fig. 12.3).

Autopsy data on old rats (144 INTACT, 56 CR and 102 HYP) of age more than 26 months revealed that 100% of INTACT rats had gross pathology and the percentage was reduced to 72% in CR and 58% in HYP (Everitt et al. 1980; Everitt and Meites 1989). The percentage of INTACT rats with gross tumors at autopsy was 67% and this was reduced by CR to 24% and by HYP to 12% (Everitt and Meites 1989). Thus HYP had greater inhibitory effects than CR on the development of these pathologies in old age.

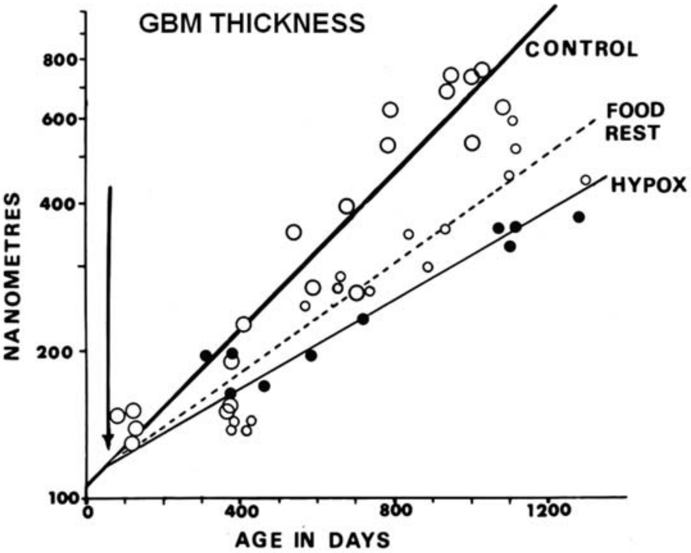

Fig. 12.2 The effect of hypophysectomy (HYPOX) and food restriction (FOOD REST) starting at 50 days on age-related thickening of glomerular basement membrane measured in nanometers in intact control (CONTROL) male Wistar rats. Hypophysectomy had a significantly greater inhibitory action than food restriction on aging in the glomerulus. HYP and FR rats consumed the same amount of food (Adapted from Wyndham et al. 1987)

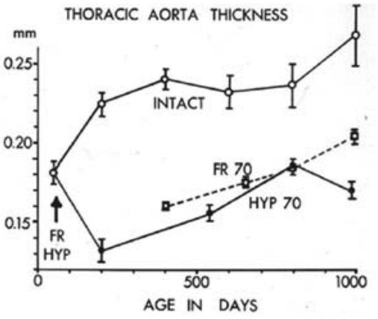

Fig. 12.3 The effect of hypophysectomy (HYP 70) and food restriction (FR 70) starting at 70 days on age-related thickening of the thoracic aorta measured in millimetres in intact control (INTACT) male Wistar rats. HYP and FR rats consumed the same amount of food. Hypophysectomy had a significantly greater inhibitory action than food restriction on aging of the aorta

The lifespan of HYP rats is shorter than that of INTACT rats (Fig. 12.4). However, when HYP rats received weekly injections of 1 mg cortisone starting at age 60 days (day of operation) they lived as long as intact rats or even longer (Everitt et al. 1980). Food restricted rats on the same food intake as HYP rats had a similar life expectancy as HYP.CORT rats. Obviously cortisone prolongs life in the HYP conventional male Wistar rat. Such studies indicate that ACTH secreted by the pituitary and glucocorticoids from the adrenal cortex extend life in these rats.

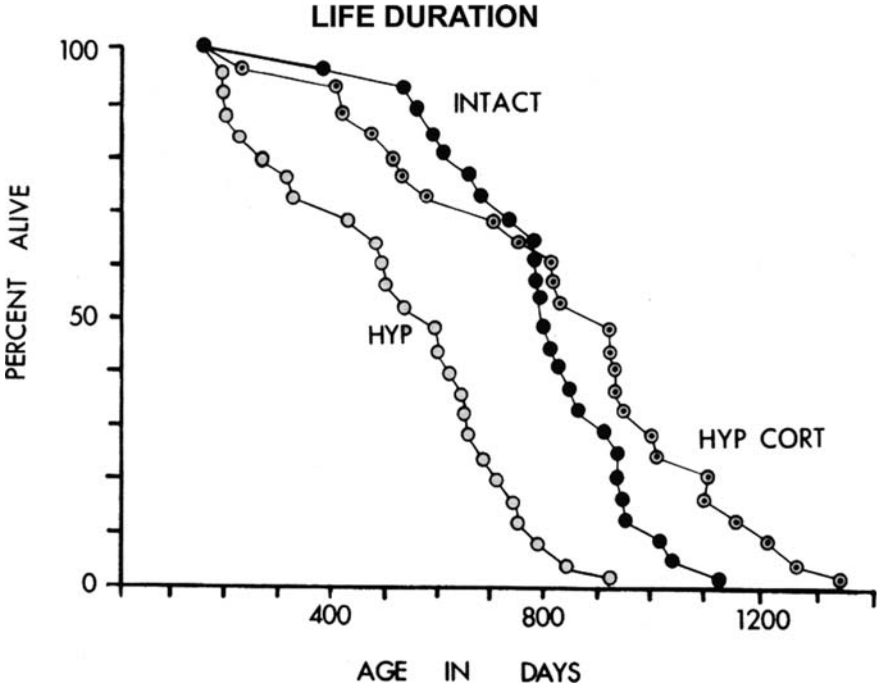

Fig. 12.4 The effect of hypophysectomy (HYP) at 70 days on the life duration of intact (INTACT) male Wistar rats. Hypophysectomized rats receiving I mg cortisone acetate per week injected subcutaneously (HYP.CORT) lived significantly longer than INTACT rats and also FR rats HYP and FR rats consumed the same amount of food (Adapted from Everitt et al. 1980)

Arking (2006) makes the point that removal of the pituitary gland from young rodents appears to preserve good health in old age when control animals are senescent. According to Arking hypophysectomy retards senescence in collagen fiber strength, immune functions, kidney diseases, oxygen consumption, neuron degeneration, vascular changes, tumor development, and wound healing.

High blood levels of glucocorticoids are found in food-restricted rats that are long-lived (Masoro 1998). These hormones are secreted under conditions of stress and give support to the hormesis theory of aging (Rattan 2008). Mild stress as in food restriction is believed to stimulate protective mechanisms in cells which prolong life. However, other neuroendocrine responses to CR may influence aging and longevity (Mobbs et al. 2001).

12.5 Hypophysectomy, Genetic Pituitary-Hormone Deficiency and Aging in the Mouse

In the mouse, hypophysectomy has been reported to reduce mean and maximum longevities and to reverse immunological decline with age (Harrison et al. 1982). Significantly, a recent study reported life extension after hypophysectomy in adult mice (Powers et al. 2006).

Genetic dwarf mice which are deficient in certain pituitary hormones are long lived (Brown-Borg et al. 1996; Bartke et al. 2001a,b; Flurkey et al. 2002). This shows that genetic factors as well as hormones are playing a role in determining lifespan (Bartke et al. 2001a,b; Mobbs et al. 2001). Mutant mice with defects in growth hormone production live longer (Flurkey et al. 2001). Genetic mutations in the mouse that interfere with growth hormone biosynthesis and actions or sensitivity to IGF-1 lead to extended longevity (Bartke et al. 1998; Bartke 2005). Hypopituitary Ames dwarf (Prop 1^{df}) and GH receptor knockout (GHRKO) mice live 35–70% longer than their normal littermates (Bartke et al. 2008). Even in rodents with life-prolonging mutations, as in the Ames dwarf mice, exposure to 30% CR starting at 2 months leads to further extension of both mean and maximum lifespan (Bartke et al. 2008).

12.6 Specific Anterior Pituitary Hormones and Aging

12.6.1 Growth Hormone

As mentioned in the previous section, animals that exhibit growth hormone deficiency or GH resistance live significantly longer than those with normal circulating GH levels. Ames and Snell dwarf mice as well as “Little” mice lack plasma GH and outlive their wild type counterparts while GH receptor knockout mice (GHRKO) are GH resistant and live longer than normal mice with intact GH receptors (mean and maximum lifespan; Brown-Borg et al. 1996; Flurkey et al. 2001; Coschigano et al. 2000). “Little” mice exhibit a mutation in the GH releasing hormone receptor and are thus unable to stimulate GH synthesis and release (Eicher and Beamer 1976; Donahue and Beamer 1993). The Ames and Snell mice lack plasma GH as well as prolactin and thyrotropin because of inappropriate differentiation of the pituitary gland during development (Bartke 1979). GHRKO mice were genetically engineered to exhibit dysfunctional GH receptors and are therefore unable to respond to GH appropriately (Zhou et al. 1997). In general, each of these mutants exhibits signs of delayed aging, lower levels of age-related disease and lifespan extension that are consistent with GH deficiency. Specifically, dwarf mice exhibit delays in sexual maturation, tumor development, age-dependent collagen cross-linking, age-dependent immune indices as well as reduced tumor incidence and resistance to tumor growth, less osteoarthritis, maintenance of cognitive function, and overall, less oxidative damage. These differences result in less age-related pathology in comparison to normal, wild type mice (reviewed in Bartke and Brown-Borg 2004). In addition, the reduced GH/IGF-1 signaling results in lower circulating glucose and insulin levels and significantly enhanced insulin sensitivity in GH deficient and GH resistant mice (Borg et al. 1995; Coschigano et al. 2003). In contrast, overexpression of GH exacerbates normal aging processes. In rodents, high circulating levels of GH are associated with signs of both premature and accelerated aging including early expression of severe glomerulonephritis and glomerulosclerosis,

Table 12.1 The effects of anterior pituitary hormones on lifespan and age-related pathology

	Reduced or absent		Increased or overexpressed	
Anterior pituitary hormones	Effect on lifespan	Effect on age-related pathology	Effect on lifespan	Effect on age-related pathology
Growth hormone	↑↑	↓↓	↓↓	↑↑
Prolactin	–	NR	–	NR
Thyroid stimulating hormone	↑	NR	↓	NR
Luteinizing hormone	NR	NR	NR	↑↑
Follicle stimulating hormone	NR	NR	NR	NR
Adrenocorticotrophic hormone	↓	NR	↑	NR

↑ increased, ↓ decreased, – no effect, NR not reported.

significant mammary and liver tumors, scoliosis, weight loss, reproductive senescence, increased astrogliosis and declines in cognitive function at much younger ages (recent review – Brown-Borg 2009) (Table 12.1).

12.6.2 Thyroid Stimulating Hormone

There is evidence that thyroid stimulating hormone or thyrotropin and its downstream effectors, mainly L-thyroxine (T4) and 3,5,3′ triiodothyronine (T3), regulate longevity. These hormones are known for their role in regulating intermediary metabolism. The most direct results of their role in aging stems from studies by Ooka and coworkers (1983, 1986). The induction of hypothyroidism in young rats led to a 4-month lifespan extension over that of euthyroid animals. Hyperthyroidism, on the other hand, resulted in a shortening of lifespan by 3-months when compared to normal rats. Vergara and coworkers (2004) showed that short-term replacement of T4 in dwarf mice had no effect on life span, however, lifelong thyroxine administration shortened lifespan by 17% compared to non-treated dwarf mice. The T4 treated mice still lived significantly longer than normal, wild type mice (Vergara et al. 2004). TSH and T4 play significant roles in metabolism and thus alter aging processes and affect mammalian longevity.

12.6.3 Prolactin

Prolactin likely plays a lesser role in aging and longevity. Ames and Snell dwarf mice lack prolactin while the GHRKO mice are hyperprolactinemic, in each case, lifespan is enhanced when compared to wild type mice with normal plasma prolactin levels. In Ames dwarf mice, prolactin replacement via pituitary transplantation somewhat shortened lifespan while this same procedure in Snell dwarf mice did not alter longevity (Bartke, unpublished observations; Flurkey et al. 2001). In agreement, prolactin deficiency in mice produces negligible effects on metabolism (LaPensee et al. 2006).

12.6.4 Gonadotropic Hormones

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) have been more recently implicated in the regulation of aging. These two pituitary hormones regulate the synthesis and secretion of estrogen and testosterone and therefore, promote growth and development of the reproductive system early in life. Sex steroids have been shown to provide important trophic and protective support for normal brain function (McEwen 2001). However, as the sex steroids decline with aging (dramatically in females at menopause), LH and FSH rise. A relatively new theory that is gaining momentum suggests that the gonadotropins are key players in hormonal modulation of aging in sexually reproductive organisms (Bowen and Atwood 2004). It is thought that the rise in LH and FSH drive the incidence and progression of age-related disease. Transgenic overexpression of LH produces mice with significant aging-related pathologies in a variety of systems and early declines in cognitive function (Mann et al. 2003; Casadesus et al. 2007). In contrast, old LH receptor knock out mice (with high circulating plasma LH) do not appear to falter in cognitive function tests when compared to age-matched wild type mice (Casadesus et al. 2007), suggesting that a functional LH receptor may be, in part, responsible for cognitive decline with aging. In addition, administration of leuprolide acetate (suppresses LH and FSH secretion via gonadotropin releasing hormone desensitization) to mice decreases brain beta amyloid concentrations (Bowen et al. 2004). Much less is known regarding the role of FSH in age-related brain disease pathology as neurons appear to lack FSH receptors and therefore, FSH effects have not been closely studied (Bowen et al. 2004). Overall, the evidence is mounting in support of a major role of LH in the pathogenesis of Alzheimer's disease and potentially other age-related neurodegenerative diseases (Bowen et al. 2004; Webber et al. 2006; Webber et al. 2007).

12.6.5 Adrenocorticotropic Hormone (ACTH)

The role of ACTH, as described earlier, is linked more directly to longevity and aging via downstream plasma corticosteroid levels and stress responses. In 1965, Friedman and coworkers showed that lifespan was prolonged in rats by high adrenal steroids and low ACTH levels. Han and coworkers (1995) showed that the reduced pituitary and plasma ACTH of CR rats may be the consequence of their elevated plasma corticosterone levels. Others suggest that the higher corticosteroid levels prime defense mechanisms and thus lead to greater stress resistance. Recent evidence showed that centenarians have high plasma levels of cortisol, CRH and ACTH potentially indicative of an activation of the entire stress axis to counteract the systemic inflammatory processes that occur with age. This observation fits with the hypothesis that lifelong low-intensity stressors activate defense mechanisms, favoring healthy aging and longevity (Masoro 2007; Genedani et al. 2008). As an added note, dwarf mice exhibit elevated corticosteroid concentrations (Borg et al. 1995) but the levels of pituitary and plasma ACTH have not been reported.

12.7 Interactions of Growth Hormone-Related Mutations with Calorie Restriction

Various phenotypic characteristics shared by the hypophysectomy, Ames and Snell dwarf mice and growth hormone GH receptor knockout (GHRKO) mice resemble the phenotype of genetically normal (wild type) animals subjected to chronic calorie restriction (CR). These characteristics include reductions in growth rate and adult body size, body temperature, levels of insulin, glucose and insulin-like growth factor 1 (IGF-1), delayed puberty and reduced fertility as well as delayed onset and reduced severity of cancer and other age-related disease. It is well documented that CR can delay aging, postpone age-related disease and extend life in mice as well as other species. Therefore, similarity of phenotypic effects of hypopituitarism, GH-resistance and CR brought up a possibility that the endocrine effects in long-lived mutant mice produce a state of "physiological CR" or metabolic adaptations similar to those produced by reduced energy intake. Comparison of the profiles of gene expression, the ratios of fat to lean mass and other characteristics of dwarf mice fed ad libitum (AL) and normal animals from the same stocks subjected to CR indicated that the long-lived GH-deficient and GH-resistant mutants are not simply CR mimetics (Mattison et al. 2000; Bartke and Brown-Borg 2004). However, considerable overlap of their phenotypes raised intriguing questions whether these mutations and CR extend lifespan by the same mechanisms, and how animals genetically endowed with characteristics resembling the effects of CR will respond to this dietary intervention.

12.7.1 CR Effects in Ames Dwarf and GHRKO Mice

To address these questions, Ames dwarf, GHRKO mice and normal animals from the same stocks were subjected to identical regimen of CR, namely reduction of daily intake of standard rodent diet by 30% gradually introduced at approximately 2 months of age and continued for the rest of their lives (Fig. 12.5).

In normal siblings of Ames dwarf and GHRKO mice, CR produced the expected significant extension of median, average and maximal lifespan in both sexes. The

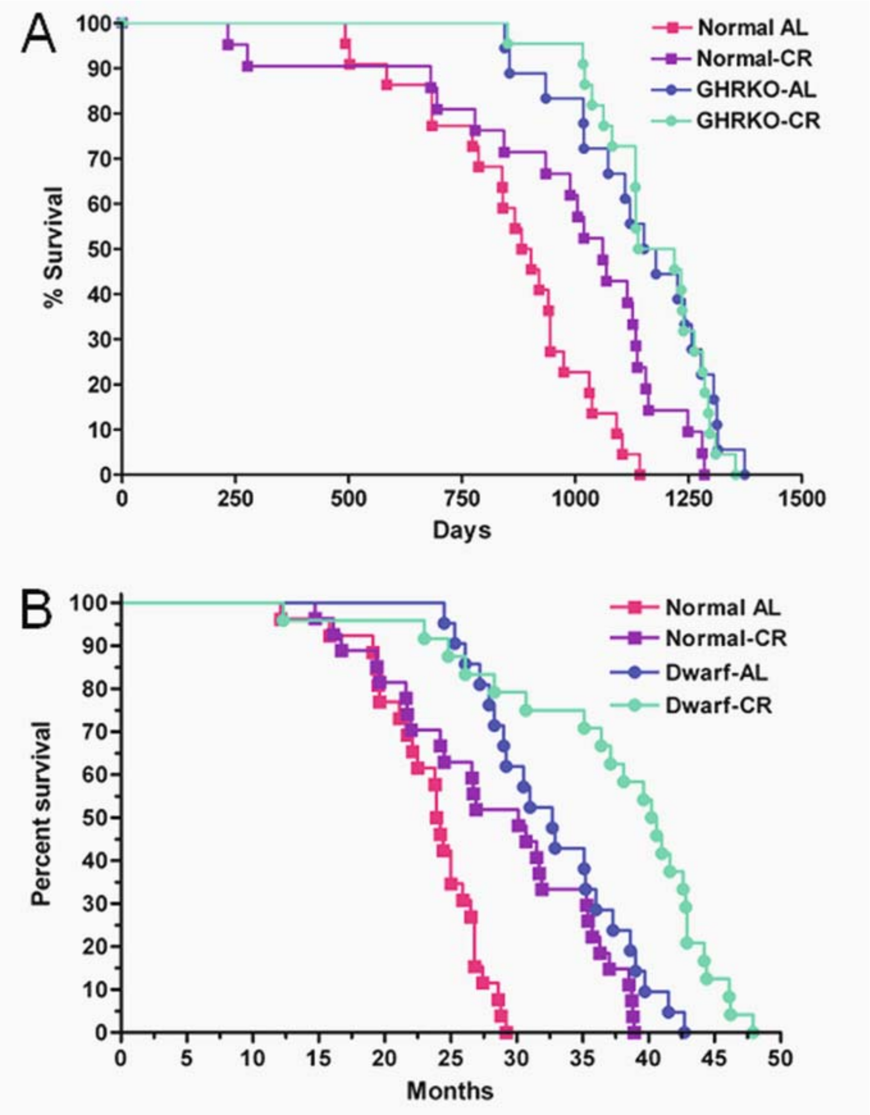

Fig. 12.5 Response of GHR-KO and Ames dwarf mice to calorie restriction. (**a**). Kaplan-Meier survival plot of normal and GHRKO mice that were fed AL or subjected to 30% CR starting at 2 months of age and maintained for the remainder of lifespan. Data from males and females are combined. (**b**). Kaplan-Meier survival plot of Ames dwarf mice that were fed AL or subjected to 30% CR starting at 2 months of age and maintained for the remainder of lifespan

results obtained in long-lived mutants were unexpected. Ames dwarf mice subjected to CR lived significantly longer than those with unlimited access to food. Both average and maximal longevity were increased regardless of gender (Bartke et al. 2001c). In contrast, in GHRKO mice an identical regimen of CR produced no alterations in the longevity of males and no changes in average or median longevity of females, while maximal female longevity was modestly increased (Bonkowski et al. 2006). Interpretation of these findings is challenging. One possible conclusion would be that GH resistance and CR extend life by the same mechanism(s), while the mechanism of life extension in hypopituitary, GH-deficient Ames dwarf mice is substantially different. These results could also imply that the GH receptor is required for CR to increase longevity, while an absence of GH does not interfere with the effectiveness of CR, or that the consequences of absent GH signaling are somehow counteracted by prolactin deficiency and/or profound hypothyroidism (endocrine defects that co-exist with GH deficiency in Ames dwarf mice). When one considers the substantial overlap of phenotypes of GHRKO and Ames dwarf mice and the comparable extension of longevity in these mutants under standard laboratory conditions (i.e., unlimited access to food), the possible conclusions listed above appear unlikely to account for their differential response to CR.

12.7.2 Insulin Signaling

Searching for a more plausible explanation of these findings, we directed our attention to alterations in insulin signaling. Changes in the insulin signaling are believed to be importantly involved in mediating the effects of GH deficiency and GH resistance on aging. Both Ames dwarf and GHRKO mice are hypoinsulinemic with reduced or "low normal" blood glucose levels, implying enhanced insulin sensitivity (Borg et al. 1995; Coschigano et al. 2003). Reduction of plasma insulin levels is more pronounced in GHRKO than in Ames dwarf mice (Borg et al. 1995; Coschigano et al. 2003). Suppression of circulating insulin levels and improved (increased) sensitivity to insulin are among the most consistent responses to CR across species and strains and are believed to contribute to delayed aging of CR animals (Masoro 2001; Roth et al. 2002). Evaluation of insulin sensitivity by insulin tolerance tests revealed that 30% CR increased the response to injected insulin in female and male normal and Ames dwarf mice but failed to enhance this response in GHRKO animals of either sex (Bonkowski et al. 2006; Giani et al. 2008). On the basis of these observations, we concluded that the failure of CR to increase longevity in GHRKO mice is associated with and likely causally related to the inability of this treatment to further enhance the extreme insulin sensitivity of these animals (Bonkowski et al. 2006).

12.7.3 CR and Gene Expression Related to Insulin Signaling

To identify molecular mechanisms responsible for the differential impact of CR on insulin signaling in GHRKO and normal mice, we have examined the effects of CR on the expression of genes related to insulin signaling, on the levels of the corresponding proteins and on their activation in response to an acute insulin stimulus in these animals. In the skeletal muscle, phosphorylation of the insulin receptor and Akt, (a key kinase which mediates transmission of the insulin signal), and the levels of Glut4, (the main glucose transporter in this tissue), were greater in GHRKO than in normal mice and were increased by CR in normal, but importantly not in the GHRKO animals (Al-Regaiey et al. 2007). Conversely the levels of activated JNK and mTOR, molecules that promote insulin resistance by inhibitory (Serine 307) phosphorylation of insulin receptor substrate 1 (IRS1), were lower in GHRKO than in normal mice and further reduced by CR only in normal animals (Al-Regaiey et al. 2007: Bonkowski dissertation 2008). Enhanced activation of insulin signaling intermediates in GHRKO as compared to normal animals and in CR as compared to AL in normal, but not GHRKO mice was detected also in the liver and in the heart (Al-Regaiey et al. 2005; Masternak et al. 2006). These results confirm differential impact of CR on whole-animal insulin sensitivity in GHRKO as compared to normal mice and identify specific steps in the insulin signaling pathway that may account for these differences. It is hoped that further studies of the interaction of murine longevity genes with CR will allow identification of mechanisms by which reduced somatotropic signaling and reduced food intake impact aging and longevity. In particular, these studies should define the role of altered secretions and actions of insulin in mediating the effects of GH and diet on healthspan and lifespan.

12.8 Conclusions

Hormones produced by the anterior pituitary gland in mammals have been shown to affect a wide variety of aging processes and ultimately, lifespan. It is important to keep in mind that the endocrine system is highly integrated so changes in one factor can lead to alterations in other hormones (i.e. GH expression is dependent on thyroid hormones). In addition, multiple pituitary hormones contribute to complex physiological functions such as growth (GH and thyroid hormones), reproduction (LH, FSH, prolactin, GH), mitochondrial function and stress resistance (thyroid hormones, ACTH, insulin, estrogen, GH), all of which impinge on aging processes. Hypophysectomy with concomitant glucocorticoid supplementation leads to reduction in age-related pathologies and extension in lifespan. Calorie restriction alters hormone expression significantly, resulting in delays in aging and increased longevity. Overall, there is abundant evidence that pituitary hormones are key regulators of aging processes.

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Chapter 13
Hormesis as a Mechanism for the Anti-Aging Effects of Calorie Restriction

Suresh I.S. Rattan and Dino Demirovic

13.1 Introduction

Chronic, intermittent or periodic calorie restriction (CR) brings about a wide range of biological effects in various organisms, including slowing down of aging and extension of lifespan. Several mechanisms have been suggested to explain the multiple biological effects of CR, which include a reduction in the levels of molecular damage due to reduced metabolism, a reduction in body temperature, alteration in the extent of cell proliferation and cell death, a decline in responsiveness to hormones, and changes in oncogenic expression leading to reduced carcinogenesis (Masoro 2006; Cavallini et al. 2008). Another mechanism that has been invoked in order to understand and explain the beneficial effects of CR is that of mild stress-induced hormesis, which stimulates maintenance and repair systems (MARS). Here we describe the phenomenon of hormesis in the context of aging and anti-aging, and review the data with respect to the view that CR is a hormetic anti-aging intervention.

13.2 Homeostasis, Homeodynamics and Aging

A fundamental characteristic of animate systems is their intrinsic ability to respond, to counteract, and to adapt to the external and internal sources of disturbance. The traditional conceptual model to describe this characteristic is *homeostasis,* which has now been shown to be incomplete and insufficient to fully describe the complexities of the living systems. The main reason for the incompleteness of the homeostasis model is its defining principle of "stability through constancy", which does not take into account the new themes, such as cybernetics, control theory, catastrophe theory, chaos theory, information and interaction networks, that comprise and

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underlie the modern biology of complexity. Therefore, since 1990s, the term *homeodynamics* (Yates 1994), is being increasingly used, which accounts for the fact that the internal milieu of complex biological systems is not permanently fixed, is not at equilibrium, and is in a dynamic regulation and interaction among various levels of organization.

Various molecular, cellular and physiological MARS are well known, and these include nuclear and mitochondrial DNA repair, free radical quenching and counteraction, protein turnover and repair, detoxification, immune response and stress responses. All these processes involve numerous genes whose products and their interactions give rise to a "homeodynamic space" or the "buffering capacity", which is the ultimate determinant of an individual's chance and ability to survive and maintain a healthy state (Rattan 2006, 2008b). Aging is a progressive shrinkage of the homeodynamic space due to the occurrence and accumulation of molecular damage, which makes an individual more vulnerable and prone to physiological imbalance. Thus, aging, age-related diseases, and eventual death are the final manifestations of unsuccessful homeodynamics and of failure of MARS (Holliday 2007; Rattan 2006, 2008b).

A critical component of the homeodynamic property of living systems is their capacity to respond to stress. In this context, the term "stress" is defined as a signal generated by any physical, chemical or biological factor (stressor), which in a living system initiates a series of biological events that enable it to counteract, adapt and survive. Table 13.1 gives a list of main molecular stress responses (SR), and are integral to the organismic property of homeodynamics. Based on the involvement of one or more molecular SR, higher order (cellular, organ level and body level) SR are manifested, which include apoptosis, inflammation, and hyperadrenocorticism leading to increased levels of circulating corticosterones in the body.

Not all pathways of the SR respond to every stressor, and although there may be some overlap, generally SR pathways are quite specific. The specificity of the response is mostly determined by the nature of the damage induced by the stressor and the variety of downstream effectors involved. For example, cytoplasmic induction of protein denaturation by heat, heavy metals and antibiotics will initiate the so-called heat shock response (HSR) by inducing the synthesis of heat shock proteins (HSP) followed by the activation of proteasome-mediated protein degradation (Verbeke et al. 2001; Liberek et al. 2008). But, unfolded proteins in the endoplasmic reticulum (ER) will induce unfolded protein response (UPR), and will initiate the induction of synthesis of a totally different set of proteins and their downstream effectors (Banhegyi et al. 2007; Yoshida 2007).

However, often the source of activation (stressor) cannot be easily identified, and may involve more than one stressor and their effectors. Examples of such SR include early inflammatory SR and neuroendocrinal SR, which lead to the synthesis and release of interleukins and corticoid hormones, respectively. Similarly, pathways involving NF-κB, Nrf2, FOXO, sirtuins and heme-oxygenase (HO) activation may involve more than one type of stressors and stress signals, including pro-oxidants, free radicals, reactive oxygen species (ROS), and nutritional components. Yet, an optimal SR is an essential aspect of successful homeodynamics and continued survival.

Table 13.1 Major molecular level stress responses

Response	Stressors	Effectors	Reference
Heat shock response	Heat, heavy metals, antibiotics, protein denaturation	Heat shock proteins, proteasome and other proteases	(Liberek et al. 2008)
Unfolded protein response	Unfolded and misfolded proteins in endoplasmic reticulum	Chaperones, co-chaperones	(Yoshida, 2007)
Autophagic response	Food starvation, hypoxia, damaged organelles	Lysosomes	(Yen and Klionsky 2008)
DNA-repair response	Radiation, oxidants, free radicals	DNA-repair enzymes	(Hakem 2008)
Antioxidant response	Free radicals, reactive oxygen species, pro-oxidants	Nrf-2, heme-oxygenase, FOXO	(Ishii et al. 2002)
Sirtuin response	Energy depletion	Sirtuins	(Longo 2009)
NF-κB inflammatory response	Pathogens, allergens, damaged macromolecules	cytokines, nitric oxide synthase	(Medzhitov 2008)

13.3 Stress and Hormesis

The consequences of SR can be both harmful and beneficial depending both on the intensity, duration and frequency of the stress, and on the price paid in terms of energy utilisation and other metabolic disturbances. But the most important aspect of SR is that it is not monotonic with respect to the dose of the stressor, rather it is almost always characterized by a nonlinear biphasic relationship. Several meta-analyses performed on a large number of papers published in the fields of toxicology, pharmacology, medicine, and radiation biology have led to the conclusion that the most fundamental shape of the dose response is neither threshold nor linear, but is U- or inverted U-shaped, depending on the endpoint being measured (Calabrese et al. 2007; Calabrese 2008). This phenomenon of biphasic dose response was termed as hormesis (Southam and Ehrlich 1943).

Since several terms such as, autoprotection, heteroprotection, adaptive response, preconditioning, hormesis, xenohormesis and others, have been used to describe the biological responses to various stressors, recommendations have been made for the use of a common terminology which is consistent with the quantitative features of the dose-response and underlying molecular foundations (for historical development of the term, see (Calabrese 2002)). It has been proposed that a common terminology should include the operational term hormesis, which would be preceded by the type of inducing agent and whether or not conditioning was present (Calabrese et al. 2007). Three main categories of such terms are:

(1) physiological conditioning hormesis, in which an exposure to a stressful condition, such as hypoxia, ischemia, radiation or a toxic chemical, conditions the system to tolerate much higher doses of the same condition subsequently;
(2) physiological/chemical/radiation hormesis: when hormesis occurs without prior conditioning; and
(3) post-exposure conditioning hormesis: The post-conditioning hormesis in which exposure to high doses of a stressor, such as radiation for cancer therapy, is followed by repeated low dose radiation exposure.

The key conceptual features of hormesis are the disruption of homeodynamics, the modest overcompensation, the reestablishment of homeodynamics and the adaptive nature of the process. An example of stress-induced hormesis is the well documented beneficial effects of moderate exercise as a hormetic agent, which initially increases the production of free radicals, acids and aldehydes (Alessio and Hagerman 2006; Ji et al. 2006; Radak et al. 2008). Another frequent observation in studies of hormesis is that a single hormetic agent, such as heat shock or physical activity, can improve the overall homeodynamics of cells and enhance other activities such as tolerance to other stresses, by initiating a cascade of processes resulting in a biological amplification and eventual beneficial effects (Mattson 2008b; Rattan 2008a). Hormesis in aging research and anti-aging interventions is represented by mild stress-induced stimulation of protective mechanisms in cells and organisms resulting in biologically beneficial effects (Le Bourg and Rattan 2008; Rattan 2008a).

13.4 Calorie Restriction as a Hormetic Agent

Based on the observations that diurnal maxima of corticosterone stress hormone concentration was higher in dietary restricted rats, it was proposed that CR acted through hormesis (Masoro and Austad 1996). Daily periods of moderate hyperadrenocorticism were considered to be protective against damage-inducing agents by restricting the energy intake. CR as a hormetic agent was further supported by the observations that CR resulted in a decrease in the body weight of animals, and this had multiple metabolic and physiological beneficial effects (Turturro et al. 1998, 2000). Additionally, corticosterone treatment was shown to modulate the level of oxidative stress in proteins and mitochondrial DNA in male Wistar rat livers, which was similar to the effects of CR (Caro et al. 2007). In the case of human beings too, an increase in the levels of corticosteroids and a loss of body weight have been observed during short-term and long-term dietary restriction (Walford et al. 2002; Yu 2006; Fontana 2009). Thus, at the whole body physiological level, CR is a sustained low intensity stressor (Masoro 2007).

However, it is important to know what other hormetic pathways may be involved in the causation of anti-aging and other health beneficial effects of CR. What follows is the review of published data on the effects of CR on various SR pathways and their effectors, as listed in the Table 13.1.

13.4.1 Heat Shock Response (HSR)

It is a universal and primordial SR achieved by the activation of the HS transcription factor(s), followed by the preferential synthesis of several HSPs, and is considered to be one of the important markers of ensuing hormesis. HS-induced anti-aging and life prolonging hormetic effects are well documented in various model systems including insects, nematodes, rodents, and human cells in culture (Rattan 2008a). Therefore, in order to consider CR to be hormetic, it is important to know if CR induces HSR. Indeed, basal levels of HSP70 have been reported to be higher in the brains of old male Wistar rats under CR than those in freely fed animals (Unno et al. 2000). Similarly, life long CR of male Fischer rats resulted in a 4-fold higher level of HSP90 and an accompanying increase in the activity of its effector, proteasome, in the soleus muscle (Selsby et al. 2005). Increased proteasomal activities have also been reported in CR Fischer rats (Shibatani et al. 1996; Shibatani and Ward 1996), and in CR yeast (Hahn et al. 2004).

Levels of HSP70 are also reported to increase in hippocampal neurones in CR Sprague-Dawley rats (Guo et al. 2000; Arumugum et al. 2006). It was also reported that hepatocytes isolated from old CR Fischer 344 rats maintained HSR in terms of transcription and translation of HSP70 after exposure to HS, as compared with the loss of HSR in freely fed old animals (Heydari and Richardson 1992). Activation of HSR was also reported for glucose-starved yeast with increased longevity (Hahn et al. 2004). Thus, activation and maintenance of HSR by CR is a strong evidence in favour of the view that CR is hormetic. However, it is not clear how exactly CR initiates HSR, and what is the exact nature of the CR-induced damage, such as protein denaturation and aggregation, which are the normally required intracellular signals for HSR.

13.4.2 Unfolded Protein Response (UPR)

Accumulation of misfolded proteins in the ER leads to the so-called ER stress response, also known as UPR, resulting in the synthesis, activation and translocation of several chaperones. UPR-indued stress proteins include GRP78/BiP, GRP94/gp96, GRP170/ORP150, GRP58/ERp57, PDI, ERp72, calnexin, calreticulin, EDEM, Herp and other co-chaperones (Lin et al. 2007; Ni and Lee 2007; Yoshida 2007; Banhegyi et al. 2007). In addition, there are mitochondrial-specific SR in mammalian cells, and involves the induction and activation of various other chaperones, such as chaperonin-10 (Cpn10/Hsp10), chaperonin-60 (Cpn60/Hsp60), and mortalin (Zhao et al. 2002; Kaul et al. 2007). An age-related decline in UPR-induced proteins and other chaperones has been reported in the neural retina of Brown Norway rats, which is prevented by CR, and the high levels of ER chaperones are maintained (Li et al. 2004). Furthermore, transient methylglyoxal production during periods of glycolysis in dietary restricted animals induces modification of the chaperone and anti-apoptotic protein HSP27, and increases its protective functions, suggesting a possible hormetic response (Hipkiss 2007).

13.4.3 Autophagy Response

Autophagy response is the lysosome-mediated and chaperone-mediated sequestering of damaged membranes and organalles, which is a SR induced during nutritional limitation, starvation, and hypoxia (Martinez-Vicente et al. 2005; Terman et al. 2007; Yen and Klionsky 2008). Several studies have shown that intermittent or chronic CR is an inducer of autophagy in the nematode *Caenorhabditis elegans,* fruitflies, and rats and mice (Bergamini et al. 2003; Cavallini et al. 2008; Cuervo 2008; Dröge 2004; Meléndez et al. 2003; Hansen et al. 2008; Ravikumar et al. 2006). Furthermore, down regulation of the nutrient sensor TOR (target of rapamycin) also elicits autophagy and extends lifespan of *C. elegans,* while inhibition of autophagy genes (ATG) eliminates the life extending effects of CR (Hansen et al. 2008). Similarly, feeding-defective *eat-2* mutants of *C. elegans* have increased autophagy and a longer lifespan, while abolishing autophagy in ATG mutants eliminates the life extending effects of reduced dietary intake (Morck and Pilon 2006). Promoting the expression of an autophagy gene, *Atg8a,* in the brains of adult *Drosophila* extended their average lifespan (Simonsen et al. 2008). Enhanced autophagy was also observed in periodic low serum-related anti-aging effects in human skin fibroblasts in culture (Rattan 2007).

13.4.4 Antioxidant Response

An important mechanism by which cells responds to oxidative stress is through the regulation of transcription of several antioxidant genes. The main regulator of this specific antioxidant phenotype is the nuclear factor-erythroid-2 (Nrf2) transcription factor, which regulates the basal and inducible expression of numerous detoxifying and antioxidant genes (Ishii et al. 2002). Under normal conditions, Nrf2 is held in the cytoplasm by the specific inhibitory protein KEAP1. Oxidative modification of cysteine residues of KEAP1 induces conformational changes and a loss of Nrf-2 binding, allowing Nrf2 to translocate to the nucleus where it heterodimerizes with specific co-factors, and leads to the transcription of various genes through the regulatory regions of antioxidant response elements (Motohashi and Yamamoto 2004; Yamamoto et al. 2004). Some of the genes activated by stress-induced activation of

Nrf2 are heme-oxygenase1 (HO-1), NAD(P)H-quinone oxidoreductase-1 (NQO1), and glutathione S-transferases (GSTs).

CR has been shown to increase the levels of Nrf2-regulated HO-1, NQO1 and GST mRNA and proteins in rodent tissues (Calabrese et al. 2008; Pearson et al. 2008). The so-called mimetics of CR, including resveratrol and curcumin, also act through Nrf2-dependent pathways (Calabrese et al. 2008; Pearson et al. 2008). In the case of *C. elegans,* transcription factor SKN-1, which is a homologue of Nrf2, gets induced by CR and activates the gene *skn-1* in a pair of nutrition sensing neurons, considered to be responsible for CR-induced extension in lifespan (Bishop and Guarente 2007).

13.4.5 DNA Repair Response

An activation of DNA repair enzymes in response to DNA damage is a SR, which is essential for the maintenance of genomic stability (Hakem 2008; Vijg 2008). Earlier studies had shown that unscheduled DNA synthesis (UDS) induced by UV irradiation was significantly higher both in the hepatocytes and kidney cells isolated from CR Fischer F344 rats (Weraarchakul et al. 1989), and in the skin cells isolated from Brown Norway x Fischer 344 F1 hybrid BNF rats (Lipman et al. 1989). Similarly, several other reports showed that DNA repair activity was maintained at higher levels in CR organisms, including yeast, *C. elgans,* rodents and humans (Haley-Zitlin and Richardson 1993; Guo et al. 1998; Raji et al. 1998; Lee et al. 1999; Howitz et al. 2003; Um et al. 2003; Cohen et al. 2004). However, these studies provide only indirect evidence that CR may be hormetic in terms of increased DNA repair ability at all ages.

Direct evidence that CR upregulates DNA repair enzyme activities at transcriptional and translation level comes from studies on young male Fischer 344 rats in which a significant increase in base excision repair, and a corresponding increase in β-pol protein and mRNA was observed after a short term CR (Cabelof et al. 2003). Furthermore, there was also an increase in the levels of DNA damage check point protein p53, which is also a sign of effective SR (Cabelof et al. 2003). However, it is not clear what kind of DNA damage signal is initiated by CR that leads to the stimulation of DNA repair activities.

13.4.6 Sirtuin Response

Activation of sirtuins causing deacetylation of histones and other proteins is considered to be a kind of SR to reduced levels of metabolic energy (North and Sinclair 2007; Longo 2009). Since an independent chapter in this book deals in detail with the role of sirtuins in CR, here we only emphasize the fact that CR-induced activation of sirtuins, and its consequential beneficial effects, including the down regulation of insulin and IGF-1 pathways, can be considered as hormetic (North and Sinclair 2007; Longo 2009).

13.4.7 NF-κB Inflammatory Response

Both acute and chronic inflammations are protective SR mechanisms in the wake of cell and tissue injury (Njemini et al. 2004; Medzhitov 2008; Vasto et al. 2009). One of the main inflammatory mediators is the transcription factor NF-κB, which together with other downstream mediators, such as TNF-α, cytokines, nitric oxide synthase (NOS), and prostaglandins, have important homeostatic functions, including repair of tissues, control of metabolism, and regulation of the hypothalamus–pituitary axis (Medzhitov 2008). However, this inflammatory SR, over a longer time scale becomes chronic inflammation, which is considered to be one of the major causes of aging and age-related diseases (Franceschi et al. 2000).

Activation of NF-κB during acute bout of exercise and leading to low level inflammation has been considered as a hormetic response (Ji et al. 2006). Other hormetic agents which stimulate NF-κB include various phytochemicals, such as curcumin and resveratrol (Calabrese et al. 2008; Mattson 2008a). However, as regards CR being a hormetic agent working through inflammatory response, there is no evidence so far that CR is pro-inflammatory even for a short while. In contrast to this, there are several reports that show the anti-inflammatory effects of CR in terms of various molecular markers of inflammation, including inactivation of NF-κB (Chung et al. 2002; Kim et al. 2002; Witte et al. 2009).

13.5 So, Is CR Hormetic?

In order to justify that hormesis is a mode in bringing about the anti-aging and life prolonging effects of CR, it is important to show that CR is a mild stressor that initiates one or more stress responses. Various lines of evidence reviewed above clearly show that CR induces several, if not all, stress responses; and that the observed biological effects in terms of enhanced maintenance and repair systems fit perfectly well with the hormetic theory of the mode of action of CR.

One of the requirements for any agent to be called hormetic is that it should act as a stressor by causing a disturbance in homeodynamics at the physiological, cellular or molecular levels. At the whole body physiological level, CR via glucose-sensing neurons and pancreatic cells initiates a series of neuroendocrine processes which lead to the reduction of IGF-1 and an increase of corticosterones (Klebanov et al. 1995; Koubova and Guarante 2003). At the tissue and cell level, CR appears to elicit SR by transiently increasing the production of ROS, which then leads to increased oxidative damage to proteins and DNA. There is some evidence that CR increases oxygen consumption and mitochondrial respiration, for example in yeast (Lin et al. 2002), and in *C. elegans* accompanied by a 3-fold increase in ROS formation (Schulz et al. 2007). Increased production of free radicals during short-term fasting has also been reported for Wistar rat liver mitochondria (Marczuk-Krynicka et al. 2003; Sorensen et al. 2006). There is also some evidence that the protein carbonyl content, a sign of oxidative damage to proteins, was significantly increased in Fischer F344 rats exposed to CR for 2 months (Judge et al. 2004). However, when measured in long-term CR animals, ROS levels and oxidatively damaged protein levels are either decreased or not different from freely fed animals (López-Torres et al. 2002; Gomez et al. 2007; Caro et al. 2008). Therefore, further studies are needed to establish the effects of other regimens of CR, such as intermittent total fasting, intermittent partial fasting, every-other-day feeding and so on.

Finally, it is clear that there are two phases of CR: an immediate adaptive response through hormesis, and a steady state and life long response in terms of improved MARS (Koubova and Guarante 2003). So far, most of the data on the phenomenological and mechanistic aspects have been collected after longer periods of CR. However, it is the immediate responses to CR which best qualify to be considered as hormetic. For example, an exposure to CR or nutritional deprivation induces a metabolic shift from the production of ATP through glycolytic pathways to mitochondrial pathways (Schulz et al. 2007), which then leads to increased respiration, increased production of ROS, and consequent damage to mitochondria, other organelles, and to macromolecules. This sequence of events then leads to compensatory hormetic responses including HSR, autophagy, DNA repair response, and antioxidant responses.

CR-induced hormesis results in an overall improvement in homeodynamics by strengthening the networks of MARS, and by reducing the rate of occurrence and accumulation of macromolecular damage. Therefore, periodic bouts of complete or partial CR, which initiate a series of protective stress responses and induce hormesis, may be also applicable to human beings as an effective anti-aging intervention.

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Part III
Calorie Restriction in the Clinical Setting

Chapter 14
Calorie Restriction and Obesity

Krista A. Varady

14.1 Introduction

Overweight and obesity are major global health concerns owing to the abundance of calorie-dense foods and the predominance of sedentary lifestyles (Bray 2008). As the prevalence of overweight and obesity increase, as does the prevalence of certain obesity related disorders, including coronary heart disease (CHD) and type 2 diabetes (Haffner 2006). Losing weight by means of dietary restriction has been shown to improve indicators of disease risk (Bray 2008). The major form of dietary restriction currently implemented is daily calorie restriction (CR). CR regimens consist of decreasing energy intake by 15–40% of baseline needs every day. Another dietary restriction regimen employed, although far less commonly, is alternate day fasting (ADF) (Varady and Hellerstein 2007). ADF regimens generally include a “feed day” where food is consumed ad-libitum over a 24-h period, alternated with a “fast day”, where food intake is either completely or partially reduced for 24 h (Varady et al. 2009).

Excess fat content in adipose tissue is strongly associated with the development of CHD and type 2 diabetes (Haffner 2006). Although the mechanisms remain unclear, recent evidence suggests that the secretion of hormones by adipose tissue may provide a link between obesity and disease risk (Fantuzzi and Mazzone 2007; Rasouli and Kern 2008). Adiponectin is a fat-cell derived hormone that exerts cardio-protective and insulin-sensitizing effects (Hajer et al. 2008; Wozniak et al. 2008). Leptin, resistin and tumor necrosis factor-α (TNF-α), in contrast, are adipocyte-derived mediators that exhibit pro-atherogenic properties (Hajer et al. 2008; Wozniak et al. 2008). Plasma adiponectin levels increase in response to weight loss, while leptin, resistin, and TNF-α levels decrease in response to weight loss (Hajer et al. 2008; Wozniak et al. 2008). Circulating concentrations of these hormones are also dictated by regional fat distribution (Rodriguez et al. 2007).

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Viscerally obese individuals have higher circulating levels of leptin and resistin and lower levels of adiponectin (Rodriguez et al. 2007), relative to subcutaneously obese individuals. The ability of CR versus ADF to favorably modulate each of these indicators of adipose biology has yet to be compared. Accordingly, the objective of this review was to evaluate and compare the effects of CR versus ADF on weight loss, visceral fat mass reduction, and adipokine profile improvement in overweight and obese humans.

14.2 Calorie Restriction Versus Alternate Day Fasting for Weight Loss

Presently, 32% of Americans are categorized as overweight (body mass index (BMI) 69% between 25.0 and 29.9 kg/m^2) while 34% are categorized as obese (BMI >30.0 kg/m^2) (Centers for Disease Control 2009). Carrying this extra weight puts these individuals at an increased risk for obesity-related disorders, such as coronary heart disease (CHD) and type 2 diabetes (Haffner 2006). Decreasing body weight by modulating diet has been shown to improve biomarkers for disease risk (Galani and Schneider 2007). For instance, plasma lipid levels (total cholesterol, LDL cholesterol, and triglycerides) generally decrease once 5% weight loss in achieved (Di Buono et al. 1999; Melanson et al. 2003). Systolic and diastolic blood pressure are also lowered once an individual reduces their body weight by 5% from baseline (Neter et al. 2003). Circulating concentrations of C-reactive protein (CRP), an extremely sensitive marker of inflammation that is positively associated with the occurrence of atherothrombotic events, is only decreased when a 10% weight loss is attained (Ryan and Nicklas 2004). As for insulin and glucose levels, a 5% reduction in body weight produces significant reductions in these biomarkers of disease risk in both overweight and obese adults (Claessens et al. 2009). CR and ADF are two forms of diet therapy that are implemented to help individuals lose weight. Table 14.1 summarizes the effects of CR and ADF on body weight in both short- and long-term human trials.

14.2.1 Effect of Calorie Restriction on Body Weight

We identified 19 CR trials (Alvarez et al. 2005; Fujioka et al. 1991; Gower et al. 2002; Janssen and Ross 1999; Kochx et al. 1999; Leenen et al. 1993; Lovejoy et al. 1995; Okura et al. 2005, 2003; Pasquali et al. 2000; Purnell et al. 2000; Rice et al. 1999; Ross et al. 2004; Tchernof et al. 2002; Thong et al. 2000; Tiikkainen et al. 2003; van der Kooy et al. 1993; Weinsier et al. 2001; Weits et al. 1989) that resulted in significant declines in body weight over 8–56 weeks in overweight and obese men and 94 women. When the studies were ordered according to treatment duration, it was observed that short-term CR trials (8–12 weeks) produced a mean weight loss ranging from 5 to 14%, moderate-term trials (13–21 weeks) resulted in weight loss

Table 14.1 CR versus ADF for weight loss and visceral fat mass reduction in overweight and obese adults

					Effect of CR/ADF on body fat distribution	
Reference	Subjects	BMI	Trial length (weeks)	Weight loss (%)	Subcutaneous fat loss (%)	Visceral fat loss (%)
CR trials						
Fujioka et al. 1991	*n*=26 Females	Obese	8	↓14	↓23	↓33
Thong et al. 2000	*n*=14 Males	Obese	12	↓12	↓16	↓25
Weitz et al. 1989	*n*=20 Females	Obese	12	↓5	↓11	↓15
Ross et al. 2000	*n*=14 Males	Obese	13	↓8	↓16	↓28
Lovejoy et al. 1995	*n*=10 Males	Obese	13	↓4	↓9	↓17
Purnell et al. 2000	*n*=21 Males	Obese	13	↓10	↓23	↓24
Leenen et al. 1993	*n*=33 Females	Obese	13	↓13	↓30	↓32
Alvarez et al. 2005	*n*=6 Males	Overweight	13	↓8	↓17	↓24
Kockx et al. 1999	*n*=25 Females	Obese	13	↓13	↓30	↓33
van der kooy et al. 1993	*n*=38 Males	Obese	13	↓15	↓35	↓39
Okura et al. 2005	*n*=34 Females	Overweight	14	↓12	↓25	↓34
Okura et al. 2003	*n*=35 Females	Overweight	14	↓11	↓19	↓15
Rice and Ross et al. 1999	*n*=9 Males	Obese	16	↓12	↓25	↓35
Janssen et al. 1999	*n*=13 Females	Obese	16	↓11	↓19	↓29
Tiikkainen et al. 2003	*n*=23 Females	Obese	21	↓10	↓14	↓19
Weinsier et al. 2001	*n*=23 Females	Overweight	26	↓17	↓41	↓38
Gower et al. 2002	*n*=18 Females	Overweight	26	↓17	↓33	↓40
Pasquali et al. 2000	*n*=10 Females	Obese	26	↓17	↓31	↓39
Tchernof et al. 2002	*n*=13 Females	Obese	56	↓15	↓28	↓38
ADF trials						
Halberg et al. 2005	*n*=8 Males	Overweight	2	↓1	–	–

Table 14.1 (continued)

Reference	Subjects	BMI	Trial length (weeks)	Weight loss (%)	Effect of CR/ADF on body fat distribution: Subcutaneous fat loss (%)	Effect of CR/ADF on body fat distribution: Visceral fat loss (%)
Heilbronn et al. 2005	n=16 Females Males	Overweight	3	↓4	–	–
Johnson et al. 2007	n=10 Females Males	Overweight	8	↓8	–	–
Varady et al. 2009	n=16 Females Males	Overweight	12	↓9	↓17	↓23

ø: No effect defined as significance $p > 0.05$, ADF: Alternate-day fasting, BMI; body mass index (BMI of 25–29.9 kg/m^2 is classified as overweight, BMI of >30 kg/m^2 is classified as obese), CR: Calorie restriction.

ranging from 8 to 15%, and long-term trials (26–56 weeks) produced weight loss ranging from 15 to 17% from baseline. Thus, longer treatment durations appear to result in greater weight loss.

However, percent weight loss may plateau after 26 weeks of treatment, as no additional reduction in body weight was observed after 56 weeks of diet (Tchernof et al. 2002). Interestingly, even the short-term trials of CR (13–21 weeks) were able to produce a minimal weight loss of 5% from baseline. As such, even short durations of CR may help overweight and obese individuals improve biomarkers of disease risk that are beneficially modulated when 5% weight loss is achieved (i.e. plasma lipids, blood pressure, insulin, and glucose levels). However in order to improve biomarkers that require a minimum weight loss of 10% (i.e. CRP levels) longer durations of treatment, ranging from 13 to 21 weeks, may be required. From the studies reviewed here, it appears as though percent weight loss is similar in men and women over equal durations of treatment. Moreover, there was no difference observed for weight loss between overweight and obese individuals. For example, overweight women in the study by Gower (Gower et al. 2002), lost the same percent of weight (17% from baseline) as obese women in the study by Pasquali (Pasquali et al. 2000), after the same duration of treatment (26 weeks).

14.2.2 Effect of Alternate Day Fasting on Body Weight

Since ADF is implemented far less frequently than CR, only four human studies (Halberg et al. 2005; Heilbronn et al. 2005; Johnson et al. 2007; Varady et al. 2009) have examined the effect of ADF on weight reduction in this population. Findings from these trials reveal that ADF is able to produce significant declines in body weight after very short trial durations. More specifically, after only 2 and 3 weeks of ADF, overweight men and women lost 1 and 4%, respectively, of their initial body weight (Halberg et al. 2005; Heilbronn et al. 2005). When treatment duration was extended to 8–12 weeks, reductions in body weight became more pronounced (8–9% weight loss, respectively, from baseline) (Johnson et al. 2007; Varady et al. 2009). Although the body of evidence is quite limited, these findings indicate that similar degrees of weight loss can be achieved by ADF compared to CR after short-term intervention periods. Additionally, since 8 and 12 weeks of ADF results in a weight loss that exceeds the 5% threshold required to modulate key biomarkers of disease, ADF may be equally as effective as CR in modulating disease risk. More human trials testing the effects of ADF on these endpoints are required before solid conclusions can be reached.

14.3 Calorie Restriction Versus Alternate Day Fasting for Visceral Fat Reduction

Upper body obesity (i.e. visceral or android obesity), as determined by increased waist circumference and elevated intra-abdominal fat area, is associated with elevated risk of CHD and type 2 diabetes (Rodriguez et al. 2007). In contrast, individuals who store comparable amounts of adipose tissue below the waistline, or in the subcutaneous gluteofemoral depots, exhibit lower risk of these obesity-related disorders (Rodriguez et al. 2007). Evidence for the pathogenic role of visceral obesity involves increased lipolysis rates and augmented free fatty acid flux from visceral fat cells (Fantuzzi and Mazzone 2007; Rodriguez et al. 2007). Owing to the proximity of visceral fat to the liver, the augmented release of these free fatty acids promotes hepatic insulin resistance and dyslipidemia associated with the obese state (Fantuzzi et al. 2007; Rodriguez et al. 2007). Thus, a key biological role of visceral fat in the development of CHD and type 2 diabetes has become apparent (Fantuzzi et al. 2007; Rodriguez et al. 2007). As such, a preferential loss of visceral fat by diet therapies may be considered metabolically advantageous. The ability of CR and ADF diets to reduce visceral fat mass is summarized in Table 14.1.

14.3.1 Effect of Calorie Restriction on Visceral Fat Mass

The effect of CR on visceral and subcutaneous fat mass was evaluated in each of the 19 trials (Alvarez et al. 2005; Fujioka et al. 1991; Gower et al. 2002; Janssen and Ross 1999; Kochx et al. 1999; Leenen et al. 1993; Lovejoy et al. 1995; Okura et al. 2005, 2003; Pasquali et al. 2000; Purnell et al. 2000; Rice et al. 1999; Ross et al. 2004; Tchernof et al. 2002; Thong et al. 2000; Tiikkainen et al. 2003; van der Kooy et al. 1993; Weinsier et al. 2001; Weits et al. 1989) reviewed in the previous section. For each study, it was observed that as percent weight loss increased, percent loss in visceral and subcutaneous mass was also augmented in a roughly linear fashion. It was also noted that there was a greater loss in percentage of visceral fat, relative to subcutaneous fat, in the majority of these studies (Alvarez et al. 2005; Fujioka et al. 1991; Gower et al. 2002; Janssen et al. 1999; Kochx et al. 1999; Leenen et al. 1993; Lovejoy et al. 1995; Okura et al. 2005, 2003; Pasquali et al. 2000; Purnell et al. 2000; Rice et al. 1999; Ross et al. 2004; Tchernof et al. 2002; Thong et al. 2000; Tiikkainen et al. 2003; van der Kooy et al. 1993; Weits et al. 1989), with a few exceptions (Okura et al. 2003; Weinsier et al. 2001). Specifically, loss of visceral fat generally exceeded that of subcutaneous fat by 5–10% in most of the study reviewed here. A possible explanation for the preferential loss of visceral fat during CR diets may involve visceral adipocytes being the primary providers of energy during times of moderate energy restriction. As for sex based differences, no apparent discrepancies between men and women were noted for this parameter.

14.3.2 Effect of Alternate Day Fasting on Visceral Fat Mass

Only one trial conducted by our lab has examined the effect of ADF on regional fat distribution (Varady et al. 2009). In this study, overweight men and women consumed 25% of their caloric needs on the "fast day" and ate ad libitum on the "feed day". After 12 weeks of diet, subjects decreased their body weight by 9% from baseline. This degree of weight loss was associated with a 23% reduction in visceral fat mass, and a 17% decrease in subcutaneous fat mass. Thus, as with CR, ADF diets produce a preferential loss of visceral fat. Moreover, results of a subanalysis revealed that there was no difference in visceral fat mass reduction between men and women in this study (Varady et al. 2009). Whether these effects of ADF on regional adiposity can be reproduced in obese individuals, will be an important focus of future research in this area.

14.4 Calorie Restriction Versus Alternate Day Fasting for Adipokine Profile Improvement

At present, over 25 fat cell-derived hormones and mediators have been discovered. Of these, adiponectin, leptin, resistin, and TNF-α, have received considerable attention. Adiponectin, which is synthesized primarily in adipose tissue, exerts both insulin sensitizing and anti-atherogenic effects (Hopkins et al. 2007). Circulating levels of adiponectin are inversely related to body weight, total cholesterol, LDL cholesterol, triglycerides, and CRP concentrations (Hopkins et al. 2007). Leptin levels, in contrast to adiponectin, are elevated in overweight individuals, and decrease when fat mass is lost (Martin et al. 2008). Although leptin is primarily known for its role in regulating food intake and energy homeostasis, this hormone may also exhibit pro-atherogenic properties by increasing platelet aggregation and arterial thrombosis (Martin et al. 2008). Resistin, another adipokine that is elevated in overweight humans, accelerates atherosclerosis by facilitating lipid accumulation in macrophages, thereby promoting the formation of foam cells (Aquilante et al. 2008). Elevated concentrations of resistin have been correlated with increased concentrations of triglycerides and CRP (Aquilante et al. 2008). TNF-α, which is released by macrophages in adipocytes, is augmented in relation to increased fat mass, and has been reported to alter insulin signaling in both cultured cells and in vivo (Hotamisligil et al. 1993). The effect of CR and ADF on plasma levels and expression of adiponectin, leptin, resistin, and TNF-α is reported in Table 14.2.

14.4.1 Effect of Calorie Restriction on Adipokine Concentrations

The ability of CR to modulate adiponectin has been tested in six human trials to date (Anderlova et al. 2006; Garaulet et al. 2004; Lejeune et al. 2007; Salas- Salvado et al. 2006; Weiss et al. 2006; Xydakis et al. 2004). These studies report that 3–6 weeks of CR, corresponding to body weight reductions between 7 and 12% in obese individuals, has no effect on circulating adiponectin concentrations (Anderlova et al. 2006; Garaulet et al. 2004; Lejeune et al. 2007; Salas-Salvado et al. 2006; Weiss et al. 2006; Xydakis et al. 2004). Adiponectin levels only increase in response to weight loss that result in BMI <29. This may explain the lack of effect in the studies examining obese subjects (Anderlova et al. 2006; Garaulet et al. 2004; Xydakis et al. 2004). In the study by Weiss (Weiss et al. 2006), however, a significant increase in adiponectin was noted in response to 52 weeks of treatment. In this long-term study, this increase in plasma adiponectin was linked to an improvement in insulin action and glucose tolerance (Weiss et al. 2006). In terms of adiponectin gene expression in adipose tissue, increases have been demonstrated in response to 6 weeks of CR in obese subjects (Garaulet et al. 2004; Salas-Salvado et al. 2006). This increase in adiponectin expression was not observed after 4 weeks of CR, however (Garaulet et al. 2004).

As for circulating leptin, levels of this adipokine appear to be highly responsive to decreases in daily energy intake even over the short term (Anderlova et al. 2006; Fogteloo et al. 2003; Garaulet et al. 2004; Kok et al. 2005; Labayen et al. 2004; Lejeune et al. 2007; Miyawaki et al. 2002; Salas-Salvado et al. 2006; Thompson et al. 2005; Vogels and Westerterp-Plantenga 2005; Weiss et al. 2006; Xydakis et al. 2004). Reductions in leptin seem to be linearly related to decreases in body weight. More specifically, it appears that a weight reduction of at least 5% from baseline is required (Labayen et al. 2004), as a 3% decrease in body weight was reported to have no effect on plasma leptin levels (Fogteloo et al. 2003). When body weight is decreased further, i.e. by 7–14%, plasma leptin concentrations decrease by ~30–70% from baseline values.

Increased circulating concentrations of resistin and TNF-α have been implicated in the pathophysiology of insulin resistance (Hotamisligil et al. 1993). In the few studies that have examined the effect CR on these adipokines, no change in plasma levels or expression has been observed (Anderlova et al. 2006; Salas-Salvado et al. 2006; Weiss et al. 2006; Xydakis et al. 2004). Circulating resistin in humans is

Table 14.2 CR versus ADF for adipokine profile improvement in overweight and obese adults

References	Subjects	BMI	Trial length (weeks)	Weight loss (%)	Effect of CR/ADF on adipokine mRNA and plasma levels			
					Adiponectin	Leptin	Resistin	TNF-α
CR trials								
Fogteloo et al. 2003	$n=30$ Females Males	Overweight	3	↓3	–	↔plasma	–	–
Anderlova et al. 2006	$n=31$ Females	Obese	3	↓	↔ plasma	↓36% plasma	↔plasma	–
Garaulet et al. 2004	$n=33$ Females	Obese	4	↓7	↔plasma ↔mRNA	↓56% plasma	–	–
Miyawaki et al. 2002	$n=20$ Females	Obese	4	↓7	–	↓72% plasma	–	–
Lejeune et al. 2007	$n=24$ Males	Overweight	6	↓12	↔plasma	↓72% plasma	–	–
Salas-Salvado et al. 2006	$n=39$ Females Males	Obese	6	↓9	↑7% mRNA	–	–	↔ mRNA
Vogels and Westerterp-Plantenga 2005	$n=120$ Females Males	Obese	6	↓8	–	↓65% plasma	–	–
Xydakis et al. 2004	$n=120$ Females Males	Obese	6	↓7	↔plasma	↓42% plasma	–	↔plasma
Labayen et al. 2004	$n=7$ Females	Obese	10	↓5	–	↓27% plasma	–	–

Table 14.2 (continued)

References	Subjects	BMI	Trial length (weeks)	Weight loss (%)	Effect of CR/ADF on adipokine mRNA and plasma levels			
					Adiponectin	Leptin	Resistin	TNF-α
Kok et al. 2005	*n*=11 Females Males	Obese	28	↓14	–	↓49% plasma	–	–
Thompson et al. 2005	*n*=90 Females Males	Obese	52	↓9	–	↓16% plasma	–	–
Weiss et al. 2006	*n*=46 Females Males	Overweight	52	↓8	↑2% plasma	–	–	↔plasma
ADF trials								
Halberg et al. 2005	*n*=8 Males	Overweight	2	↓1	Feed day: ↓ Fast day: ↑	Feed day: ↑ Fast day: ↓	–	↔plasma
Johnson et al. 2007	*n*=10 Females Males	Overweight	8	↓8	–	Feed day: ↑ Fast day: ↓	–	–

↔: No effect, ADF: Alternate-day fasting, BMI; body mass index (BMI of 25–29.9 kg/m^2 is classified as overweight, BMI of >30 kg/m^2 is classified as obese), CR: Calorie restriction.

primarily derived from the macrophages residing in adipocytes, rather than the adipocytes themselves. Recent evidence suggests that plasma resistin levels may be more reflective of inflammatory status, rather than body fat content (Lehrke et al. 2004). This may explain why resistin was not altered in any of the studies reviewed here.

14.4.2 Effect of Alternate Day Fasting on Adipokine Concentrations

Two human studies to date have examined the effect of ADF on circulating adipokines. Recently, Johnson (Johnson et al. 2007) demonstrated that after 8 weeks of ADF, leptin levels increased on feeding days and declined on the fasting days. A superimposed, progressive decline in leptin levels on feeding days was also observed throughout the 8-week study (Johnson et al. 2007). These effects of feeding and fasting on leptin levels were also demonstrated in the study by Halberg (Halberg et al. 2005). As for adiponectin, increased levels of this adipokine have been observed on 251 fasting days, but not feeding days. This effect was shown consistently on each fasting day throughout the 2-week study (Halberg et al. 2005). In terms of TNF-α, no effect of ADF on circulating concentrations of this cytokine was noted after 2 weeks of treatment (Halberg et al. 2005). The effect of ADF on circulating resistin levels has yet to be examined.

14.5 Conclusion

In conclusion, CR and ADF diets appear to be equally as effective in modulating several parameters of adipose biology. Specifically, both CR and ADF resulted in comparable reductions in body weight and visceral fat mass after similar treatment durations. CR and ADF also displayed an equal potency in augmenting levels of the beneficial adipokine, adiponectin, while decreasing levels of the unfavorable adipokine, leptin. These beneficial alterations in adipose tissue physiology may provide significant clinical and metabolic benefits. Thus, these findings suggest that ADF may be implemented as effective alternative to CR to help overweight and obese individuals lose weight and lower the risk of CHD and type 2 diabetes.

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Chapter 15
Caloric Restriction and Cardiovascular Disease

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15.1 Introduction

The dietary regimen known as caloric restriction (CR) can delay aging and extend lifespan in evolutionarily distant organisms, including both invertebrates (Austad 1989) and laboratory rodents (Yu et al. 1985). Cardiovascular disease and stroke are the leading causes for age-related mortality and morbidity in elderly humans (Lakata 2003; Lakatta and Levy 2003a, b), hence improving cardiovascular health and delaying the progression of atherosclerosis by CR may result in lifespan extension in humans as well. This overview focuses on emerging evidence that reactive oxygen species (ROS) and activation of inflammatory pathways each play a central role in cardiovascular aging, and discusses the potential mechanisms by which CR exerts anti-oxidative and anti-inflammatory vasoprotective effects promoting cardiovascular health. At least a portion of the age-related increase in cardiovascular morbidity and mortality appears to be secondary to increases in systemic risk factors for atherosclerosis (e.g. plasma lipid profile and glucose levels and blood pressure), which may be amenable to improvements through CR. In addition, we discuss the role of neuroendocrine mediators induced by CR, which may directly improve vascular health by enhancing cellular functions and impacting gene expression in endothelial and smooth muscle cells to create a microenvironment in the vascular wall, which does not favor atherogenesis (e.g. attenuation of ROS production, anti-inflammatory effects).

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15.2 Caloric Restriction Improves Cardiovascular Risk Factor Profile

CR was shown to attenuate atherogenesis in rodents (Guo et al. 2002). The cardiovascular effects of CR observed so far are consistent with the view that CR may confer vasoprotection in humans, although the effects of CR on progression of atherosclerosis and plaque composition in elderly humans or aged primates (Cefalu et al. 2004) are still not well documented. A significant portion of our current knowledge on the effects of CR on cardiovascular risk factors in humans emanates from studies in which obese individuals were treated with some form of relatively short-term dietary restriction to cause weight loss (Pierce et al. 2008; Miyaki et al. 2008). CR also appears to exert beneficial effects on systemic risk factors for atherosclerosis in non-obese people (Walford et al. 1992; Fontana et al. 2004; Meyer et al. 2006; Fontana et al. 2007). Accordingly, CR in both obese and in non-obese individuals elicits significant decreases in serum cholesterol, triglycerides, fasting glucose and fasting insulin levels as well as in systolic and diastolic blood pressure (Walford et al. 1992; Fontana et al. 2004, 2007; Lefevre et al. 2009).

Studies of the effects of CR in rhesus monkeys have also shown reductions in serum triglycerides (Verdery et al. 1997), lipoprotein(a) in males (Edwards et al. 2001) and fasting plasma glucose and insulin levels (Kemnitz et al. 1994), which are likely to contribute to the cardioprotective effect of CR. The data available from rodent studies are consistent with the beneficial effects of CR on cardiovascular risk factors (Maeda et al. 1985; Yu et al. 1985; Ahmet et al. 2005).

15.3 Role of Increased Bioavailability of Nitric Oxide in the Cardioprotective Effects of CR

Abundant experimental and clinical data show that aging as well as metabolic diseases associated with accelerated vascular aging (e.g. type 2 diabetes) impair endothelial NO production, which results in significant vasomotor dysfunction (recently reviewed elsewhere (Csiszar et al. 2005)). It is generally accepted that tonic release of NO from the endothelium exerts vasculoprotective and cardioprotective effects, such as maintenance of normal coronary blood flow, inhibition of both platelet aggregation and inflammatory cell adhesion to endothelial cells and disruption of pro-inflammatory cytokine-induced signaling pathways. In contrast, impaired bioavailability of NO is thought to play a role in atherogenesis. Recent data suggest that CR in laboratory rodents prevents aging-induced endothelial dysfunction by increasing the bioavailability of NO, both at the level of microcirculation and in conduit arteries (Nisoli et al. 2005; Mattagajasingh et al. 2007; Ungvari et al. 2008b; Kondo et al. 2009). Available data further suggest that weight reduction with low calorie diets improves flow-mediated vasodilation in obese individuals (Sasaki et al. 2002; Raitakari et al. 2004; Pierce et al. 2008). More data is needed to confirm that CR can also improve endothelial function in non-obese aged monkeys (Cefalu et al. 2004) and elderly humans (Meyer et al. 2006) independent of weight reduction.

The mechanisms by which CR increases the bioavailability of NO and improves endothelial function in aged rodents are likely to include up-regulation of eNOS (Ungvari et al. 2008). An interesting study by Mattagajasingh et al. (2007) recently reported that SIRT1, a molecular target of CR, deacetylates eNOS, stimulating eNOS activity. Yet, this study also raised a number of unanswered questions. Because the enzymatic de-acetylation requires protein-protein interaction, we feel that further studies are needed to confirm that SIRT1 (a nuclear enzyme) indeed can interact with eNOS (a cell membrane-associated enzyme) in vivo. Because endothelium-derived NO plays a fundamental role in the regulation of tissue metabolism (e.g. by inducing mitochondrial biogenesis (Nisoli et al. 2005)), CR-induced improvement of endothelial health and the resulting increased NO bioavailability may be directly involved in the extension of lifespan in mammals. This idea is supported by the finding that eNOS-deficient mice exhibit an accelerated aging phenotype associated with premature cardiac failure, mitochondrial dysfunction and increased mortality (Li et al. 2004; Ojaimi et al. 2005). Finally, because mitochondriopathy in the vasculature per se represents an early manifestation of endothelial dysfunction (Addabbo et al. 2009), improved NO bioavailability by CR may prevent vascular energetic dysfunction, which is likely to contribute to vascular functional alterations in aging (Csiszar et al. 2006).

15.4 Anti-inflammatory Cardiovascular Effects of CR

Atherosclerotic vascular disease is now recognized as a chronic inflammatory disease (Libby 2002). There is increasing evidence showing that aging is associated with chronic low-grade vascular inflammation, which promotes the development of cardiovascular diseases (reviewed recently elsewhere (Lakatta et al. 2003a,b)). Accordingly, aging is associated with endothelial activation, increased expression of adhesion molecules (Zou et al. 2004, 2006; Csiszar et al. 2007; Ungvari et al. 2007a) and enhanced leukocyte adhesiveness to the endothelial cells (Yang et al. 2004; You et al. 2006; Ungvari et al. 2007). In aging a pro-inflammatory shift develops in the vascular cytokine expression profile, including an age-related up-regulation of TNFα, IL-1β and IL-6 (Csiszar et al. 2003, 2004; Kim et al. 2008). All of the aforementioned factors promote a pro-inflammatory microenvironment in the vascular wall.

Previous studies have shown that endothelial activation and pro-inflammatory gene expression in aging is promoted, at least in part, by oxidative stress-induced NF-κB activation (You et al. 2006; Ungvari et al. 2007). In this regard it is interesting that CR seems to attenuate vascular NF-κB induction and endothelial activation in aged rats (You et al. 2004; Csiszar et al. 2007). CR inhibits NF-κB activation in other tissues as well, suggesting that the anti-inflammatory action of CR contributes to its general anti-aging action by preventing the development of inflammation-related diseases in multiple organ systems (Kim et al. 2008; Jung et al. 2009). In addition to inhibition of NF-κB CR is likely to disrupt other pro-inflammatory signaling pathways as well. For example, CR protected against the age-associated increase of JNK and P38 activities in aged rat aortas and reversed the age-related increase of AP-1 DNA binding activity (Castello et al. 2005). It is significant that even short-term CR may confer anti-inflammatory effects (Jung et al. 2009), suggesting that some form of adult-onset CR may play a role in the therapeutic paradigm for cardiovascular diseases.

Aging is also associated with increased circulating levels of inflammatory mediators (e.g. TNFα, IL-6 and CRP), both in humans and rodents (Ershler et al. 1993; Heilbronn and Clifton 2002; Phillips and Leeuwenburgh 2005), which are known to correlate with impaired cardiovascular function. In studies of CR in rats and mice, it was found that CR results in marked decreases in these inflammatory markers (Spaulding et al. 1997; Kalani et al. 2006). The observation that CR in humans decreases circulating CRP and TNFα (Meyer et al. 2006) provides preliminary evidence that CR may also reduce chronic low-grade inflammation in humans.

15.5 Attenuation of Cardiovascular Oxidative Stress by CR

The general concept that oxidative stress is involved in many age-related diseases, including cardiovascular disease, appears well founded. The role of increased oxidative stress in eliciting endothelial dysfunction (Tschudi et al. 1996; van der Loo et al. 2000; Csiszar et al. 2002; Adler et al. 2003; Sun et al. 2004; Francia et al. 2004) and development of chronic low-grade vascular inflammation in aging has been established (Ungvari et al. 2004; Csiszar et al. 2005). In 1996 Dr. Richard Weindruch's group (Sohal and Weindruch 1996) proposed that the anti-aging action of CR stems from the attenuation of the age-associated increase in oxidative stress (Bevilacqua et al. 2005). Indeed, there is clear evidence that CR attenuates the age-associated accumulation of oxidized lipids, proteins, and nucleic acids in multiple organ systems (Lass et al. 1998; Harper et al. 2004; Sanz et al. 2005). Accordingly, CR also effectively decreases vascular production of reactive oxygen species (ROS) in aged laboratory rodents (Ungvari et al. 2008). These data are consistent with the findings that endothelial cells obtained from CR mice exhibit decreased $O_2^{\bullet -}$ and H_2O_2 production as compared with those obtained from mice fed ad libitum (Yang et al. 2004). Previous studies also demonstrated that CR significantly attenuates oxidative DNA damage (Guo et al. 2001) and normalizes the tissue content of lipid peroxidation-derived aldehydes (HNE, MDA) in aortas of aged rats (Castello et al. 2005). There are studies linking the reduction of vascular oxidative stress to the anti-atherogenic effect of CR in $ApoE^{-/-}$ mice (Guo et al. 2002).

It has been amply demonstrated that mitochondria are a major source of the increased ROS production found in aging, both in the heart and the vasculature

(Ungvari et al. 2007, 2008). CR decreases age-related mitochondrial ROS production in most tissues studied, including blood vessels and the heart (Gredilla et al. 2001). In that regard it is significant that CR also prevents many of the age-related alterations in mitochondrial gene expression in the heart (Dhahbi et al. 2006; Linford et al. 2007; Chang et al. 2007). There are studies suggesting that in certain laboratory rat strains, some of the effects of CR on cardiac mitochondrial function and protein expression may be gender specific (Colom et al. 2007), yet it is unclear whether the same holds true in humans. The mechanisms underlying the mitochondrial protective effects of CR are likely multifaceted and may include a decrease in protonmotive force in mitochondria (Lambert and Merry 2004), up-regulation of mitochondrial antioxidant systems and induction of mitochondrial biogenesis (Civitarese et al. 2007) (which reduces the electron flow per unit mitochondria, attenuating mitochondrial ROS generation). In aged endothelial cells the precise sites of mitochondrial ROS production in the electron transport chain are still a matter of debate, but it seems that both complex I and complex III are capable of producing $O_2^{\bullet -}$, particularly when electron transport is inhibited (Han et al. 2001; Brand et al. 2004; Balaban et al. 2005; Ungvari et al. 2007). Impairment of complex IV activity would be expected to increase the fraction of upstream electron carriers in a reduced state, thereby increasing the rate of univalent reduction of oxygen and subsequently the generation of $O_2^{\bullet -}$ from the aforementioned sites. In contrast, a relative increase in complex IV expression/activity in CR would likely result in decreased mitochondrial ROS production in cardiovascular tissues. Previous studies have identified vascular NAD(P)H oxidases as another important source of ROS production in the aged vasculature (Hamilton et al. 2001; Csiszar et al. 2002; Adler et al. 2003; Csiszar et al. 2005, 2007; Park et al. 2007). Thus, future studies should elucidate how CR affects NAD(P)H oxidase-dependent ROS generation in aged blood vessels.

15.6 Role of Neuroendocrine Factors in CR-Induced Vasoprotection

The mechanisms underlying the beneficial effects of CR on mammalian healthspan are undoubtedly multifaceted, involving both cell-autonomous effects (e.g. changes in mitochondrial function), changes in paracrine regulation (e.g. altered cytokine microenvironment) and effects mediated by neuroendocrine factors. A key role of neuroendocrine factors in phenotypic responses due to CR is supported by the observations of de Cabo et al. (2003) that in vitro treatment of cultured hepatocytes with sera from CR animals mimics phenotypic effects observed in vivo during CR. Neuroendocrine mediators present in the circulation reach endothelial cells and elicit a variety of responses, and we have good reason to believe that these circulating factors mediate, at least in part, vasoprotective effects during CR. Indeed, our recent studies suggest that circulating factors triggered by CR activate endogenous cytoprotective mechanisms in endothelial cells, thus contributing to the anti-oxidative and anti-inflammatory vasoprotective effects of CR (Ungvari, Csiszar and de Cabo, submitted). In support of this point of view, our data show that circulating factors in sera of CR animals convey significant anti-inflammatory effects in cultured endothelial cells and mimic the vascular phenotypic changes induced in animals by CR in vivo. Accordingly, treatment of coronary arterial endothelial cells with sera from CR animals effectively prevents TNFα-induced NF-κB activation, induction of NF-κB-driven gene expression (including that of ICAM-1) and endothelial activation (Csiszar et al. 2009). Another potentially important finding is that treatment of cells in vitro with sera from CR animals also mimics the known anti-apoptotic effects of CR in vivo (Edwards et al. 2007) preventing the induction of apoptosis in endothelial cells by multiple pro-apoptotic stimuli. Interestingly, the CR mimetic resveratrol also confers significant anti-apoptotic effects in endothelial cells both in vitro (Ungvari et al. 2007; Csiszar et al. 2008a) and in vivo (Pearson et al. 2008a).

The actual circulating factor(s) by which the anti-oxidant, anti-apoptotic and anti-inflammatory effects of CR are mediated are presently unknown. There is increasing evidence that adiponectin, whose serum level is known to be increased by CR, both in laboratory animals (Shinmura et al. 2007, 2008; Niemann et al. 2008) and humans (Raitakari et al. 2004; Jung et al. 2008) may contribute to the cardioprotective effects of CR. Adiponectin, besides its effects on cellular metabolism, has been shown to exert anti-inflammatory effects, specifically inhibition of NF-κB activation (Ouchi et al. 2000) in endothelial cells. Recent studies reported that while CR improves the recovery of left ventricular function after ischemia/reperfusion and limits infarct size in wild-type mice, these effects are completely abrogated in adiponectin-depleted mice (Shinmura et al. 2007). Furthermore, recent evidence suggests that CR can promote revascularization in the lower extremity in response to tissue ischemia via an AMPK-eNOS-dependent mechanism that is mediated by adiponectin (Kondo et al. 2009). Adiponectin also can induce mitochondrial biogenesis (Civitarese et al. 2006), mimicking a key effect of CR. Thus, future studies are needed to further elucidate the role of adiponectin in the anti-inflammatory and anti-atherogenic effects of CR in aging.

Previous studies have attributed some of the effects of CR to a decreased insulin-like signaling. First studies in *Caenorhabditis elegans* provided evidence that neuroendocrine factors, namely, reduced insulin-like signaling, may promote longevity in lower organisms during CR. By now it is well established that insulin-like signals promote the phosphorylation and deactivation of DAF-16, a forkhead transcription factor, which is a key regulator of oxidative stress resistance and metabolism in *C. elegans* (reviewed in Tatar et al. 2003). There is also solid evidence that attenuation of IGF-like signaling contributes to longevity response to CR in *Drosophila* (Partridge et al. 2005). The first evidence to support a role of insulin-like signals in regulation of mammalian longevity came from the observation that mice with hypopituitary dwarfism (Ames dwarf, Snell dwarf) have low plasma IGF-1 levels and exhibit an extended longevity phenotype (Brown-Borg et al. 1996). However, there is increasing evidence suggesting that decreased IGF-1 signaling is unlikely to be responsible for the protective effects of CR in the cardiovascular system. First, although in calorie-restricted animals the levels of circulating IGF-1 decline 30–40%, this is not the case in humans. Recent studies clearly demonstrate that unlike in rodents, long-term severe CR does not reduce serum IGF-1 concentration or the IGF-1: IGF binding protein ratio in humans (Fontana et al. 2008). Second, there is strong evidence that IGF-1 itself is an important protective factor in the cardiovascular system. For example, aortas of Ames dwarf mice exhibit increased endothelial ROS generation and down-regulation of major anti-oxidant enzymes as compared to vessels from wild type mice (Csiszar et al. 2008a). Moreover, in cultured coronary arterial endothelial cells and cardiac myocytes, treatment with IGF-1 significantly reduces cellular $O_2^{\bullet-}$ and H_2O_2 production and ROS generation by mitochondria and up-regulates expression of antioxidant enzymes and eNOS (Csiszar et al. 2008a). These experimental findings accord with the observations that in humans GH and IGF-I deficiency is associated with premature atherosclerosis and elevated cardiovascular disease mortality (Elhadd et al. 2001). Cardiovascular disease risk is even elevated among apparently healthy individuals who have serum IGF-1 levels in the low-normal range (Roubenoff et al. 2003). There is also increasing evidence that IGF-1 may exert vasculoprotective effects in aging (Rivera et al. 2005; Lopez-Lopez et al. 2007), improving cardiac diastolic function (Groban et al. 2006) and preventing hippocampal microvascular rarefaction (Sonntag et al. 1997, 2000; Khan et al. 2002); IGF-1 was also shown to protect cardiac myocytes from apoptotic cell death (Li et al. 1997, 1999; Leri et al. 1999) and to promote cardiac stem cell survival and proliferation (Torella et al. 2004; Urbanek et al. 2005). The finding that cardiac overexpression of IGF-1 significantly improved cardiomyocyte contractile function in old mice (Li et al. 2007) supports the view that IGF-1 signaling plays a protective role in the cardiovascular system and that loss of IGF-1 contributes to cardiac aging. Thus, low IGF-1 levels are unlikely to contribute to the beneficial cardiovascular effects of CR.

15.7 Role of SIRT1 and Nrf2 in CR-Induced Vasoprotection

Multiple lines of evidence indicate that homologs of the NAD^+-dependent protein deacetylase SIRT1 mediate the lifespan extension by CR in lower organisms (Wood et al. 2004; Rogina and Helfand 2004; Wang and Tissenbaum 2006). In mammals SIRT1 is also induced by CR (Cohen et al. 2004), suggesting a central role for this enzyme in mammalian physiology and the stress response. SIRT1 is expressed in the cardiovascular system (Csiszar et al. 2008b; Shinmura et al. 2008) and is induced by CR (Shinmura et al. 2008). Because sera from CR animals and humans induces SIRT1 in many cell types (Cohen et al. 2004; Allard et al. 2008), including endothelial cells (Ungvari, Csiszar and de Cabo, submitted), it is likely that SIRT1 activation contributes to the vasoprotective effects conveyed by CR-induced neuroendocrine factors. This idea is supported by the findings that pharmacological SIRT1 activation or SIRT1 overexpression confer significant anti-oxidative and anti-inflammatory effects in cultured endothelial cells (Csiszar et al. 2008b). Moreover, endothelium-specific overexpression of SIRT1 effectively attenuates development of atherosclerosis in ApoE-deficient mice (Zhang et al. 2008).

Another emerging intracellular target for CR-induced neuroendocrine factors is Nrf2 (NF-E2-related factor 2). Nrf2 is a transcription factor that binds to the antioxidant response element (ARE) of target genes and increases the transcription of a variety of proteins involved in oxidative stress resistance and detoxification of free radicals (such as glutathione-S-transferase, NQO1 and the antioxidant enzymes glutathione reductase, glutathione peroxidase and catalase). The aforementioned Nrf2-dependent ROS detoxification systems are expressed and functional in endothelial cells. Previous studies from several laboratories have provided convincing evidence that the ARE-mediated genes are regulated by atheroprotective laminar flow through a Nrf2-dependent mechanism (Chen et al. 2003; Mostoslavsky et al. 2006; Dai et al. 2007; Warabi et al. 2007). Induction of Nrf2 in cultured endothelial cells was shown to protect the cells from H_2O_2 -mediated cytotoxicity (Mostoslavsky et al. 2006). Nrf2 also suppresses endothelial activation and inhibits monocyte adhesiveness to the endothelial cells (Mostoslavsky et al. 2006). It has been demonstrated that in tissues of the aged rat there is a significant decline in transcriptional activity of Nrf2, which causes age-related loss of glutathione synthesis (Suh et al. 2004), likely promoting cellular oxidative stress. In contrast, our recent studies demonstrated that CR is associated with induction of Nrf2-regulated genes, which are responsible for the anti-carcinogenic effects of CR (Pearson et al. 2008). In a series of studies we are testing the hypothesis whether Nrf2 induction plays a key role in CR-induced vasoprotection. Although presently it is unknown how aging and CR affect Nrf2 transcriptional activity in the vascular endothelial and smooth muscle cell, we have strong evidence for an age-dependent decline in glutathione synthesis in aged vessels, which is prevented by CR (Csiszar et al. 2009).

15.8 Conclusions

Aging is associated with oxidative stress and inflammatory changes in the vascular and cardiac transcriptome and secretome which promote the development of cardiovascular diseases. CR, both in animal models and human patients, appears to confer significant anti-oxidant and anti-inflammatory effects. Whether some form of long-term dietary restriction will play a prominent role in the therapeutic paradigm to reverse or delay the considerable aging-induced functional decline of the cardiovascular system remains a subject of current debate. Overall, we can expect that recent advances in our understanding of the mechanisms underlying the cardiovascular protective effects of CR will, in the not-so-distant future, yield novel anti-aging therapeutic approaches that will be exploited for the benefit of elderly patients.

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Chapter 16

The Effect of Caloric Restriction on Physiological, Psychological and Behavioral Outcomes in Humans: Results from *CALERIE*

Leanne M. Redman and Eric Ravussin

16.1 Caloric Restriction and Lifespan

Calorie restriction (CR) has been shown since the 1930s by McCay to retard the aging process (McCay et al. 1935), extending the median and maximal lifespan in various models and species (Walford et al. 1987). While the exact mechanisms through which CR is able to extend the lifespan have yet to be fully elucidated, CR reduces metabolic rate and oxidative damage, improves markers of age-related diseases such as insulin resistance for diabetes, and has been shown to alter neuroendocrine activities in animals (Heilbronn et al. 2006). Results from studies in rhesus monkeys suggest that prolonged CR can also oppose many age-associated pathophysiological changes including learning and behavior changes, plasma insulin concentrations and resting energy expenditure (Blanc et al. 2003; Bodkin et al. 2003; Roth et al. 2004). Since many changes associated with prolonged CR are important to the health and survival of humans, and excessive caloric intake is associated with morbidity and development of chronic diseases, it has become an important research objective to assess the feasibility, the safety and the effects of prolonged CR in well-controlled human trials.

16.2 Caloric Restriction May Alter the "Rate of Living" and Oxidative Stress

The aging process may be influenced by energy restriction through a reduction in the "rate of living" (Sacher and Duffy 1979) leading ultimately to reduced oxidative damage. An ongoing controversy among investigators appears to be whether chronic CR leads to metabolic adaptation, a reduction in the metabolic rate which is out of proportion to the diminished metabolic mass of the organism (Heilbronn

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and Ravussin 2003). Results from rats and monkeys suggest that most of the collected data should be reevaluated using appropriate methods of normalizing the metabolic rate for changes in metabolic size (Ravussin and Bogardus 1989). For example, Blanc recently calculated a 13% reduction in resting energy expenditure after adjusting for fat-free mass in an 11 year long study of energy restricted monkeys (Blanc et al. 2003). Recently, however Selman using doubly labeled water to measure total energy expenditure reported that caloric restricted rats expended 30–50% more energy than expected (Selman et al. 2005).

The "free radical theory of aging" or oxidative stress hypothesis is one of the well-supported theories of aging. It is widely accepted that the metabolic rate of an organism is a major factor in the rate of aging, and is inversely related to its lifespan (Sohal and Allen 1985). Additionally, since 1–3% of consumed oxygen is associated with the production of reactive oxygen species (ROS), namely superoxide [$(O_2^{\bullet -})$], hydrogen peroxide [$(H_2O_2^{\bullet -})$], and the hydroxyl ion ($OH^{\bullet -}$) (Alexeyev et al. 2004), the production of these highly reactive molecules from normal aerobic metabolism is also in direct proportion to an organism's metabolic rate. Many investigators have shown that modulation of the oxidative stress of an organism through prolonged CR is able to retard the aging process in various species, including mammals (Sohal and Weindruch 1996; Weindruch et al. 1986). As a result of increased oxygen consumption, aerobic exercise is associated with increased production of reactive oxygen species in muscle tissues (Fulle et al. 2004). However, exercise training boosts up the antioxidant capacity of skeletal muscle probably resulting in decreased overall oxidative stress (Sachdev and Davies 2008).

16.3 Caloric Restriction, Cardiovascular Disease, Insulin Resistance and Type 2 Diabetes Mellitus

Elevated levels of LDL, excessive reactive oxygen species generation, hypertension and diabetes are all potential causes for the development of endothelial dysfunction, a precipitating event in the progression of atherosclerosis. These factors are believed to initiate an inflammatory response in the injured endothelial tissue. Long-term CR is associated with sustained reductions in factors related to endothelial dysfunction in humans, such as decreased blood pressure (Velthuis-te Wierik et al. 1994), reduced levels of total plasma cholesterol and triglycerides (Walford et al. 2002), and reduced markers of inflammation such as C-reactive protein and plasminogen activator inhibitor type-1 (Bastard et al. 2000; Heilbronn et al. 2001; Mavri et al. 2001). A long-term CR study in humans supports the feasibility of using CR as a protective effect against atherosclerosis by showing a 40% reduction in carotid artery intima-media thickness in CR participants relative to a control group (Fontana et al. 2004).

Strong evidence shows that long-term energy restriction in lean and obese subjects improves insulin sensitivity, a mechanism by which CR may act to extend lifespan (Walford et al. 1999, 2002). Additionally, prolonged CR reduces fasting glucose and insulin concentration, two factors believed to contribute to the aging process due to protein glycation (Robertson 2004) and mitogenic action (Stenkula et al. 2004), respectively. This compelling evidence suggests that weight loss due to CR may be the most effective means of improving insulin sensitivity, thereby decreasing the risk for the development of diabetes mellitus.

16.4 Why Caloric Restriction?

Since the first report of prolonged lifespan in rodents more than 70 years ago (McCay et al. 1935) CR is gaining momentum as an intervention with the potential to ward off age associated diseases and delay death. While the first observations were reported in rodents, similar observations have been reported across a wide range of species including yeast, worms, spiders, flies, fish, mice and rats (Heilbronn and Ravussin 2003). While the effects of CR in longer lived species remain unknown, results reported thus far from 3 non-human primate colonies suggest that CR might have a similar effect in longer-lived species. While lifespan data remains inconclusive (Ingram et al. 2006), CR monkeys display a substantially reduced age-related morbidity (Bodkin et al. 2003; Lane et al. 1995). In humans, data from controlled trials is lacking and of course no long-term prospective trials of CR have been conducted with survival being the primary end-point (Heilbronn et al. 2006). There is however, a lot that can be learned from a handful of epidemiological and cross-sectional observations in longer-lived humans, centenarians and individuals who self-impose CR.

16.4.1 Centenarians from Okinawa

Probably the most intriguing epidemiological evidence supporting the role of CR in lifespan extension in humans comes from the Okinawans (Kagawa 1978). Compared to most industrialized countries, Okinawa, Japan has 4–5 times the average number of centenarians with an estimated 50 in every 100,000 people (Japan Ministry of Health 2005). Reports from the Japanese Ministry of Health, Labor and Welfare show that both the average (50th percentile) and maximum (99th percentile) lifespan are increased in Okinawans. From age 65, the expected lifespan in Okinawa is 24.1 years for women and 18.5 years for men compared to 19.3 years for women and 16.2 years for men in the USA (Willcox et al. 2006). What is interesting about this population is that a low caloric intake was reported in school children on the island more than 40 years ago and later studies confirmed a 20% CR in adults residing on Okinawa compared to mainland Japan (Suzuki et al. 2001). A recent estimate of the energy balance of a cohort of Okinawa septuagenarians during youth to middle age suggested a 10–15% energy deficit (Willcox et al. 2007). This energy deficit can be attributed to laborious occupations and daily activities as farmers and a diet that was rich in nutrients yet low in energy density (Willcox et al.

2007). Unfortunately with the increase in American presence on Okinawa in recent years (location of US military base and increased availability of fast food chains) unpublished reports suggest that the longevity-promoting lifestyle (i.e. CR and high levels of physical activity) is threatened and may be reversed.

16.4.2 The Vallejo Study

To our knowledge there is only one study that was designed to test the effects of CR without malnutrition in non-obese humans (Vallejo 1957). This was a study of alternate day feeding in 120 men whereby the 60 participants in the CR group received an average of 1,500 kcal per day for 3 years whereas the 60 others were ad libitum. This amounted to approximately 35% CR compared to the control group. While the initial report was brief, post-hoc analyses conducted several years later (Stunkard 1976) indicated that the death rate tended to be lowered in the CR group and hospital admissions were reduced in these individuals by approximately 50% (123 days for CR vs. 219 days for Control).

16.4.3 Unexpected CR in Biosphere 2

The unexpected low availability of food during the 2 year Biosphere 2 experiment provided a unique opportunity to observe the effects of CR in a group of non-obese humans (Fig. 16.1).

Biosphere 2 was an enclosed 3.15-acre ecological laboratory that housed seven ecosystems or biomes resembling the earth; rainforest, savannah, ocean, marsh, desert, and agriculture and human/animal habitats (Walford et al. 1992). For 2 years, 8 individuals including Dr. Roy Walford were completely isolated within this “mini-world” where 100% of the air and water was recycled and all the food grown inside. Due to unforeseen problems with agriculture early on, food supply became quickly insufficient. Food intake for the 8 individuals was projected at ~2,500 kcal/day and estimates from food records maintained by one of the Biospherians suggested diets were restricted by ~750 kcal/day in each person during the first 6 months. The resulting ~15% weight loss in the Biospherians was associated with many physiological, hematological, biochemical and metabolic alterations (Walford et al. 2002; Weyer et al. 2000) consistent with calorie restricted rodents and primates including reductions in insulin, core temperature and metabolic rate (Lane et al. 1995).

16.4.4 Randomized Controlled Trials of Calorie Restriction in Non-obese Humans

As for randomized controlled trials, results from a 2-year study of CR in humans is only a few years away. The National Institute on Aging (NIA) is sponsoring a trial;

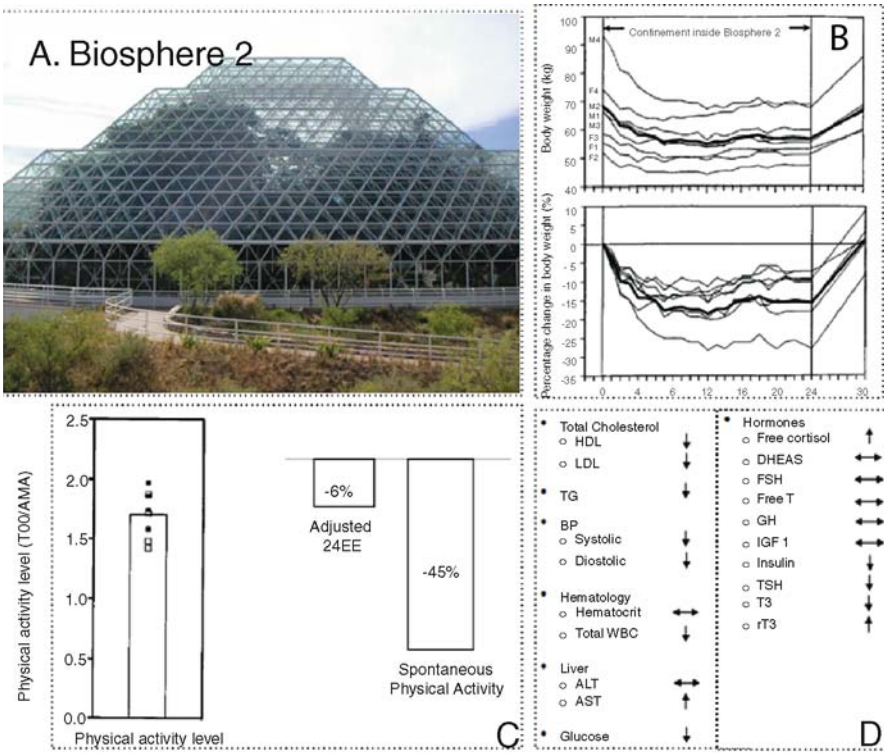

Fig. 16.1 Biosphere 2 (**Panel A**), a 3.15-acre ecological enclosure provided an unexpectedly low availability of food for 8 individuals who were housed inside for 2 years in the early 1990s. This study of the nature of calorie restriction resulted in ~15% weight loss (**Panel B**), in changes in energy expenditure and physical activity (**Panel C**) and many hematological, biochemical and metabolic alterations (**Panel D**) consistent with calorie restricted rodents and primate including reductions in insulin, core temperature and metabolic rate (Walford et al. 2002; Weyer et al. 2000)

CALERIE (Comprehensive Assessment of the Long-term Effect of Reducing Intake of Energy) which is for the first time, scientifically testing the effects of 25% CR in ~150 non-obese healthy men and women aged 20–47 (50 for men) years. Three clinical sites are involved in the trial; Washington University in St. Louis, MO, Tufts University in Boston, MA and the Pennington Biomedical Research Center in Baton Rouge, LA. The protocol and endpoints for this multi-center trial were developed from experience acquired in 3 independent Phase 1 trials conducted at each clinical site (Das et al. 2007; Heilbronn et al. 2006; Racette et al. 2006).

The remainder of this review will concentrate on the results from the Phase 1 study conducted at the Pennington Center. For 6 months, 48 men and women were randomized to one of four treatment groups (Anton et al. 2008; Civitarese et al. 2007; Larson-Meyer et al. 2006; Martin et al. 2007a, b, c; Redman et al. 2007; Williamson et al. 2007, 2008). For the CR group, individuals were restricted to 75% (a 25% CR) of their weight maintenance energy requirements assessed by doubly labeled water (Redman et al. 2009). The other groups were: (1) CR plus exercise group for which the calorie deficit was also 25% from weight maintenance but half (12.5%) was achieved by CR and half (12.5%) by increasing energy expenditure with structured aerobic exercise, (2) a low calorie diet group in which participants consumed 890 kcal/day to achieve a 15% weight loss and thereafter followed a weight maintenance diet and (3) a healthy diet control group that followed a weight maintaining diet based on the American Heart Association Step 1 diet. The effects of the calorie restriction interventions were determined from changes in various physiological and psychological endpoints after 3 and 6 months.

16.5 Physiological Effects of Caloric Restriction

Aging is considered to be either "primary", that is the inevitable deterioration of cells and tissues structure and function that occurs independent of disease, lifestyle and environmental causes or, "secondary" where the decline in tissue structure and function occurs as a result of external influences including diseases (Holloszy and Fontana 2007). Attenuation of primary aging results in an increase in maximal lifespan, whereas delays in the progression of age-related disease or secondary aging increases mostly average lifespan. Calorie restriction (CR) is the only known intervention that can slow primary aging and protect against secondary aging. Six months of CR produced favorable alterations in physiological and behavioral outcomes. Results from the first randomized study of calorie restriction (CALERIE) at the Pennington Center are discussed below:

16.5.1 Body Composition

Throughout the 6 month intervention there was a progressive decline in body weight that reached ∼10% for the CR group at the completion of the study (Redman et al. 2007). Body composition analysis by dual x-ray absorptiometry and multi-slice computed tomography showed that the loss of tissue mass was attributable to significant reductions in both fat mass (CR:–24±3%) and fat-free mass (CR:–4±1%), and a 27% decrease in both visceral and subcutaneous fat depots. It was interesting to note that the fat distribution within the abdomen was not altered by CR (Redman et al. 2007). We also observed a reduction in subcutaneous abdominal mean fat cell size by ∼20%, a lowering of hepatic lipid by ∼37% but no change in skeletal muscle lipid content (Larson-Meyer et al. 2006) (Fig. 16.2).

16.5.2 Biomarkers of Longevity

A biomarker of aging or longevity is considered to be any parameter that reflects physiological or functional age; it must undergo significant age-related changes,

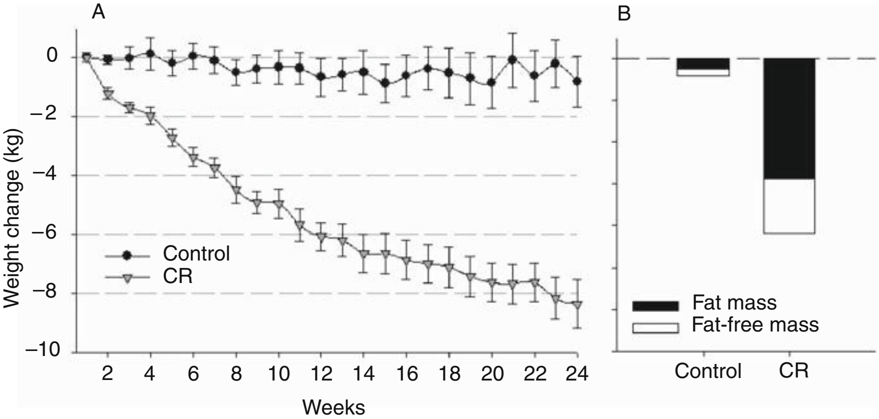

Fig. 16.2 Our 6 month study of 25% caloric restriction resulted in a progressive decline in body weight that reached ~10% the completion of the study (Redman et al. 2007). Body composition analysis by dual x-ray absorptiometry showed that the loss of tissue mass was attributable to significant reductions in both fat mass (CR: –24±3%) and fat-free mass (CR:–4±1%)

be slowed or reversed by treatments that increase longevity (e.g. calorie restriction), and must be reliably measured. Numerous biomarkers have been identified in rodents and primates including body temperature and hormones such as DHEA-S and insulin (Roth et al. 2002). In the CALERIE study, 2 out of the 3 biomarkers of longevity (Roth et al. 2002) were improved with 6 months of 25% CR (Heilbronn et al. 2006). Significant reductions were observed in both fasting insulin concentrations (–29±6%) and core body temperature (–0.20±0.05°C), whereas DHEA-S was unchanged by the intervention. These findings echo results previously reported in nonhuman primates and rodents on CR and long lived men in The Baltimore Longitudinal Study of Aging (Roth et al. 2002) (Fig. 16.3).

16.5.3 Cardiovascular and Diabetes Risk Factors

With heart disease and stroke ranked numbers one and three in the causes of death in the USA (Rosamond et al. 2007) delaying the progression of atherosclerotic cardiovascular disease maybe one potential mechanism by which CR promotes longevity. The risk factors for CVD including blood lipids, blood pressure, hemostatic factors, inflammatory markers and endothelial function are worsened with aging (Celermajer et al. 1994; Mendall et al. 1996). At least a portion of these age-related changes appear to be secondary to increases in adiposity and/or reductions in physical activity (DeSouza et al. 2000; Mora et al. 2006) and, therefore, may be amenable to improvements through prolonged caloric restriction. Six months

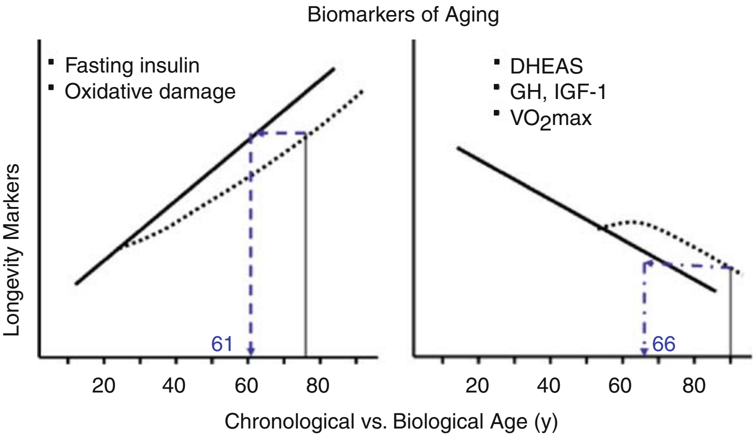

Fig. 16.3 Can calorie restriction improve biological age and extend chronological age? This figure summarizes some of the potential biomarkers of aging. It is hypothesized that calorie restriction will change the biological trajectory of these biomarkers and therefore improve biological age and extend chronological age. For example, the *left panel* shows an individual aged 75 years. With prolonged calorie restriction it is hypothesized that fasting insulin and oxidative damage will be reduced in this individual. Therefore an individual although 75 will have a biological age 14 years younger. Similarly the individual on the right at 90 years with prolonged calorie restriction will be biologically similar to an individual aged 66 years

of CR significantly reduced triacylglycerol (TG) and factor VIIc by 18 and 11%, respectively (Lefevre et al. 2009). HDL-cholesterol was increased and fibrinogen, homocysteine and endothelial function were not changed. According to total and HDL cholesterol (expressed as their ratio), systolic blood pressure, age and gender, the estimated 10-year CVD risk was 28% lower after only 6 months of CR.

Insulin resistance is an early metabolic abnormality that precedes the development of hyperglycemia, hyperlipidemia and overt type 2 diabetes. Both insulin resistance and β-cell dysfunction are associated with obesity (Forsey et al. 2003; Matsumoto et al. 2003; Utzschneider et al. 2004). Calorie restriction reduces fat mass and delays the development of age-associated diseases such as type 2 diabetes. While in obese humans, it is well established that calorie restriction and weight loss improve insulin sensitivity (Dengel et al. 1996; Niskanen et al. 1996), the effects of calorie restriction on insulin sensitivity and therefore diabetes risk is not well understood in overweight and lean individuals. In our study of 6 months CR we observed a 40% improvement in insulin sensitivity in the CR group, although this did not reach significance ($p=0.08$) (Larson-Meyer et al. 2006). The acute insulin response to glucose (AIRg), however was significantly decreased from baseline (CR: 29±7%, $p<0.01$) indicating an improvement in β-cell responsiveness to glucose.

16.5.4 Metabolic Adaptation and Oxidative Stress

One of the most popular proposed theories by which CR promotes lifespan extension is the "rate of living theory" (Sacher 1977). It is hypothesized that a lowering of the metabolic rate reduces the flux of energy with a consequential lowering of reactive oxygen species and rate of oxidative damage to vital tissues (Sohal and Weindruch 1996). Indeed, CR is associated with a robust decrease in energy metabolism, including a lowering of resting metabolic rate (or sleeping metabolic rate), lowering of the thermic effect of meals and a decrease in the energy cost of physical activity. However, as mentioned earlier, whether total energy expenditure is reduced beyond the expected level (i.e. metabolic adaptation) for the reduction in the metabolizing mass (fat-free and fat mass: FFM and FM) following CR is debated.

As expected, absolute 24-h energy expenditure and sleeping metabolic rate (both measured in a respiratory chamber) were significantly reduced from baseline with CR ($p<0.001$). Importantly however, both 24-h sedentary and sleeping energy expenditures were reduced ∼6% beyond what was expected for the loss of metabolic mass i.e. FFM and FM (Heilbronn et al. 2006). This metabolic adaptation was also observed for RMR measured by a ventilated hood indirect calorimeter (Martin et al. 2007c). These physiological responses were associated with a reduced amount of oxidative stress as measured by DNA damage. DNA damage was reduced from baseline after 6 months in CR ($p=0.0005$), but not in controls (Heilbronn et al. 2006). In addition 8-oxo7, 8-dihidro-2′deoxyguanosine (8oxodG) was also significantly reduced from baseline in CR subjects ($p<0.0001$). These data confirm findings in animals that CR reduces energy metabolism, oxidative stress to DNA, both potentially attenuating the aging process.

16.5.5 Endocrine Adaptations

Thyroid function. Short-term studies of CR in humans have reported alterations in thyroid function. Four weeks of complete fasting resulted in a decrease in triiodothyronie (T3) and an increase in reverse triiodothyronine (rT3) which was associated with a reduction in metabolic rate (Vagenakis et al. 1975). The CRONIES (a self selected group engaging on long-term CR) have significantly lower T3 but not thyroxine (T4) or thyroid stimulating hormone (TSH) concentrations compared to age-, sex- and weight-matched controls (Fontana et al. 2006).

In the CALERIE study, plasma T3 concentrations were reduced from baseline in the CR group after 3 ($p<0.01$) and 6 months ($p<0.02$) of intervention (Heilbronn et al. 2006). Similar results were found for the change in plasma T4 in response to the treatment. When the data of the subjects in the three CR groups were combined into one intervention sample, we observed significant linear relationships between the change in plasma thyroid hormones and the degree of 24-h metabolic adaptation after 3 months of intervention (T3; $r = 0.40$, $p = 0.006$ and T4; $r = 0.29$, $p=0.05$) (Heilbronn et al. 2006).

The Somatotropic Axis. Aging is marked by a reduction in both growth hormone (GH) and insulin like growth factor-1 (IGF-1) concentrations in healthy adults resulting from a reduced amount of GH secreted at each burst without alterations of burst frequency or GH half life (Veldhuis et al. 2005). Unlike rodents, weight loss via energy restriction in humans increases GH (Smith 1996). After 6 months of CR, 11-h mean GH concentrations were not changed with CR nor was the secretory dynamics in terms of the number of secretion events, secretion amplitude and secretion mass (Redman et al. 2010). The fasting plasma concentration of ghrelin, a GH secretagogue was significantly increased from baseline but IGF-1 was unaffected. Despite a significant reduction in weight and visceral fat and an improvement in insulin sensitivity, mean GH concentrations were not altered by the 6 month intervention. In agreement with this observation was the finding that both GH and IGF-1 were not affected by the chronic food shortage experienced by the individuals in Biosphere 2 (Walford et al. 2002).

DHEA-S. Given the evidence from cross-sectional (Orentreich et al. 1984) and longitudinal studies (Orentreich et al. 1992) that DHEA-S declines with age, DHEA-S is considered to be a reliable endocrine marker of human aging and longevity (Roth et al. 2004). It was hypothesized that CR will delay or attenuate the age-associated decline in DHEA-S. In our 6-month study in young individuals (37±2 years), we observed no alteration in DHEA-S (Heilbronn et al. 2006). Similarly, DHEA-S was not changed with 2 years of energy restriction in the individuals within Biosphere 2 (Walford et al. 2002). To our knowledge, there has been no report of DHEA-S levels in those individuals from the Calorie Restriction Society (CRONIES) who are self-imposing CR. The lack of agreement between the human and non-human primate data is believed to be due to first, the chronological age of the subjects at the onset of CR, and second, to the duration of CR. Young adult monkeys undergoing CR for 3–6 years had an age-related decline in DHEA-S of 3% compared to 30% in monkeys fed ad libitum (Lane et al. 1997). In contrast, CR initiated in older animals (∼22 years) did not attenuate the age-associated decline in DHEA-S (Urbanski et al. 2004). These explanations remain to be tested in longer term studies of CR in humans.

16.5.6 Physical Activity

Daily energy expenditure has three major components: resting metabolic rate (RMR), the thermic effect of food and the energy cost of physical activity. Investigation of changes in physical activity are important in studies of CR not only because the contribution of physical activity to daily energy expenditure is variable, but also because it is not known if individuals volitionally or non-volitionally decrease their level of physical activity in an attempt to conserve energy (Keys et al. 1950). In our study, we observed no change in spontaneous physical activity in a respiratory chamber (Martin et al. 2007c) consistent with earlier reports of no alterations in spontaneous physical activity (Ravussin et al. 1985) or posture allocation in obese individuals following weight loss (Levine et al. 2005). These findings are not surprising if the current hypothesis that spontaneous physical activity is biologically determined is true (Levine et al. 2005; Zurlo et al. 1992). However with a measure of energy metabolism in free-living conditions (doubly labeled water) we found that a metabolic adaptation exists after 3 months (–386±69 kcal/day) but not after 6 months of CR (Redman et al. 2009). This adaptation was evident even after total daily energy expenditure (TDEE) was adjusted for sedentary energy

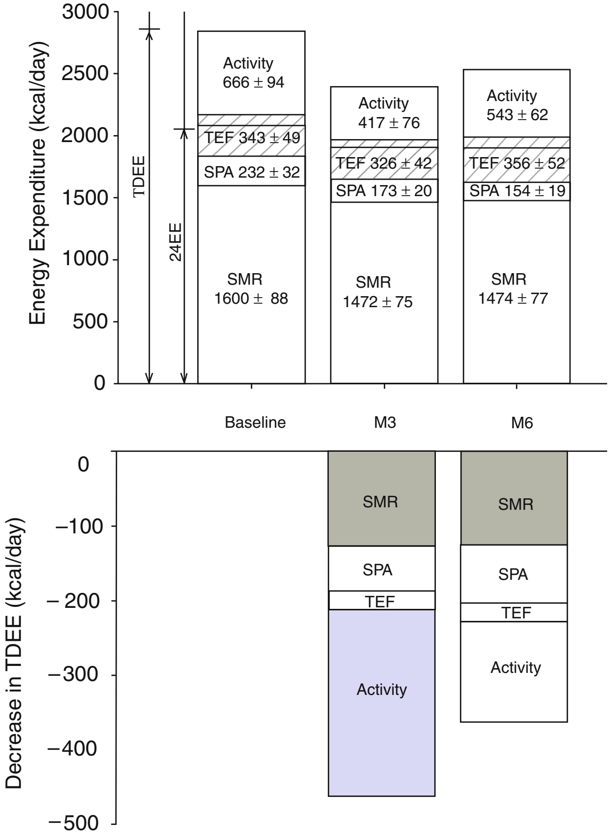

Fig. 16.4 The effect of caloric restriction on all components of daily energy expenditure (*Top Panel*). The changes in total daily energy expenditure after 3 and 6 months of CR (*Bottom Panel*) are shown and those representing a metabolic adaptation (larger than due to weight loss) are highlighted in grey (Redman et al. 2009). Combining two state of the art methods (indirect calorimetry and doubly labeled water) for quantifying precisely the complete energy expenditure response to CR in non-obese individuals, we identified a reduction in sedentary energy expenditure that was 6% larger than what could be accounted for by the loss in metabolic size, i.e. a "metabolic adaptation" (Heilbronn et al. 2006) and a metabolic adaptation in the free-living situation as well. This adaptation comprised not only a reduction in cellular respiration (energy cost of maintaining cells, organs and tissue alive) but also a decrease in free-living activity thermogenesis (behavioral adaptation)

Table 16.1 Summary of the psychological and behavioral responses to 6 months of calorie restriction in humans

Psychological/behavioral responses
Development of eating disorder symptoms
↓ Disinhibition
↓ Binge eating
↓ Concern about body size and shape
↔ Fear of fatness
↔ Purgative behavior
Depressed mood
↓ MAEDS Depression scale
↔ Beck depression inventory II
Subjective feelings of hunger
↓ Eating inventory, perceived hunger scale
Quality of life
↑ Physical functioning
↔ Vitality
Cognitive performance
↔ Verbal memory
↔ Short-term memory and retention
↔ Visual perception and memory
↔ Attention/concentration

metabolism (24-h or sleeping energy expenditure) indicating that changes in other components of daily energy expenditure mostly as physical activity and less via diet-induced thermogenesis are also involved. In support of this, physical activity level calculated by either the ratios of TDEE to RMR or sleeping metabolic rate (Martin et al. 2007c), or TDEE adjusted for sleeping metabolic rate (Redman et al. 2009), was significantly reduced at month 3 by 12% and returned toward baseline values after 6 months of intervention (Fig. 16.4). Interestingly, despite lower physical activity levels, participants reported an improvement in physical functioning, a primary component of quality of life. All the effects of caloric restriction on physiological outcomes are summarized in Table 16.1.

16.6 Psychological and Behavioral Effects of Caloric Restriction

Calorie restriction in humans might prove to have positive effects on physical health and longevity, resulting in the practice of CR or the identification of CR mimetics. Very little is known about the effect of CR on the quality of life and furthermore, if people attempt to follow CR for health promotion important questions must be answered about possible negative effects of CR on psychological well-being, cognitive functioning, mood, and subjective feelings of appetite. Determining the effect of CR on these parameters is critical to learn if adhering to a CR regimen is feasible and if CR has unintended negative consequences that would offset the potential of its health benefits. Phase I of CALERIE provided a unique opportunity to examine the effect of 6 months of CR on psychological and behavioral endpoints. We here summarize the effects of CR on the development of eating disorder symptoms, quality of life, mood (symptoms of depression), subjective ratings of appetite, and cognitive function.

16.6.1 Development of Eating Disorder Symptoms

One of the most pressing concerns about CR is the potential development of symptoms of eating disorders. This concern is based in part on the Keys study (Keys et al. 1950), which found that 50% CR for 6 months among healthy men was associated with the development of eating disorder symptoms, e.g., binge eating (Garner 1997). Additionally, CR or the intent to restrict intake has been associated with the onset of eating disorders, including anorexia (Williamson 1990) and bulimia nervosa (Polivy and Herman 1985), and binge eating disorder (Williamson and Martin 1999). Hence, there is a need to examine both the benefit and potential harm of CR in humans, particularly for people who are not obese, and to answer important safety questions before CR is recommended (Vitousek et al. 2004a; Vitousek et al. 2004b).

In our study, participants completed an assessment battery that included: (1) the Multifactorial Assessment of Eating Disorder Symptoms (MAEDS), which measures six symptom domains associated with eating disorders (binge eating, purgative behavior, depression, fear of fatness, avoidance of forbidden foods, restrictive eating) (Anderson et al. 1999), (2) the Eating Inventory, which measures dietary restraint, disinhibition, and perceived hunger (Stunkard and Messick 1988), and (3) the Body Shape Questionnaire (BSQ) (Cooper et al. 1987), which measures concern about body size and shape.

The three "dieting" groups in CALERIE, including the CR group, reported higher dietary restraint scores in comparison to the Control group at months 3 and 6, but measures of eating disorder symptoms did not increase and some decreased (Williamson et al. 2008). All groups, except the control group, reported a significant reduction in disinhibition at month 6 whereas binge eating, decreased in all groups at months 3 and 6. Concern about body size/shape decreased at 3 and 6 months among the three dieting groups but did not change in the control group. The Fear of Fatness and Purgative Behavior subscales of the MAEDS did not change during CR.

16.6.2 Subjective Feelings of Hunger

The ability of people to follow CR could be limited by feelings of increased hunger. We evaluated change in appetite ratings during CR using the perceived hunger scale of the Eating Inventory (Stunkard and Messick 1988) and the Visual Analogue Scales (VAS), which have been found to be reliable and valid measures of appetite: hunger, fullness, desire to eat, satisfaction, and prospective food consumption (Flint et al. 2000). During the 6-month study, appetite ratings changed, but the changes among the dieting groups were not different from those in the control group. Moreover, based on the perceived hunger scale of the Eating Inventory, hunger was reduced in the CR group at month 6 (Williamson et al. 2008).

16.6.3 Quality of Life and Mood

The Minnesota Semi Starvation study (Keys et al. 1950) indicated that CR can negatively affect mood, and therefore the effect of CR on mood and quality of life (QOL) becomes an important factor when considering the feasibility of CR in humans. During CALERIE Phase I, the Medical Outcomes Study Short-Form 36 Health Survey (SF-36) (Ware and Sherbourne 1992; Ware et al. 1993, 2002) was used to measure QOL, and the Beck Depression Inventory II (Beck et al. 1996) and depression scale of the MAEDS were used to measure mood. Our results indicate that depressed mood, measured by the BDI-II, did not change during the trial. Additionally, in the CR group, scores on the MAEDS depression subscale decreased at 3 and 6 months in comparison to baseline (Williamson et al. 2008). Together, the results indicate that CR had no negative effect on mood during this trial and, in fact, symptoms of depressed mood, measured with the MAEDS, decreased in the CR group.

The SF-36 was used to test the effects of CR on two components of QOL – physical functioning and vitality. All dieting groups, but not the control group, had improved physical functioning during the trial. For the CR group, physical functioning was significantly improved at both months 3 and 6 but CR had no significant effect on vitality.

16.6.4 Cognitive Function and Performance

Self-reported dieting or calorie restriction has been associated with deficits in cognitive performance (e.g., memory and concentration deficits) (Green and Rogers 1998; Kemps et al. 2005). Nevertheless, cognitive impairment is frequently mediated by preoccupation with food and body weight (Kemps and Tiggemann 2005), suggesting that obsessive thoughts about food and weight, rather than CR, negatively affect cognitive performance. If CR has negative effects on cognitive performance, the feasibility of CR in humans would be in doubt.

In our trial, cognitive performance was evaluated empirically at baseline and months 3 and 6 with a comprehensive neuropsychological battery (Martin et al. 2007a). Verbal memory was measured with the Rey Auditory and Verbal Learning Test (RAVLT) (Schmidt 1996), short-term memory and retention with the Auditory Consonant Trigram (ACT) (Peterson 1966; Peterson and Peterson 1959), visual perception and memory with the Benton Visual Retention Test (BVRT) (Sivan

1992), and attention/concentration with the Conners' Continuous Performance Test-II (CPT-II) (Conners 2000). During CR, no pattern of memory or attention/concentration deficits emerged. The degree of daily energy deficit also was not correlated with change in cognitive performance; hence, these data indicate that CR did not have a negative effect on cognitive performance (Martin et al. 2007a). All the effects of caloric restriction on psychological and behavioral outcomes are summarized in Table 16.1.

The psychological and behavioral findings from CALERIE provide important information about the feasibility and safety of CR in humans. Calorie restriction was not associated with the development of eating disorder symptoms, decreased quality of life, depressed mood, or cognitive impairment. In fact, many of these endpoints improved, and changes in subjective ratings of appetite were similar in the CR group to those of the control group. These results suggest that CR might be feasible and have few unintended consequences, at least among overweight individuals. Additional research is needed to determine the feasibility and safety of CR in other samples of the population and over a longer duration.

16.7 Could CR Increase Longevity in Humans?

The wealth of CR literature in rodents however allows us to address some important questions relating to the practicality and feasibility of CR in humans. Relevant and practical questions are; 1. How much CR do we need to improve age-related health and possibly longevity? and 2; How long do we need to sustain CR in order to obtain these benefits? Analysis of 24 published studies of CR in rodents (CR up to 55%) indicated a strong negative relationship between survival and energy intake (Merry 2000) and a positive relationship between the duration of CR and longevity. Using the prediction equations derived from the rodent data above (Merry 2000), we and others estimated that a 5-year life extension could be induced by 20% CR starting at age 25 and sustained for 52 years, i.e. the life expectancy of a male in the US. However, if a 30% CR was initiated at age 55 for the next 22 years, the gain would only be 2 months (Fig. 16.5).

Certainly there are individuals who self-impose CR with the CRON (Calorie Restriction with Optimal Nutrition) diet for health and longevity. A group of 18 CRONIES (only 3 women) have recently been studied after 3–15 years of CR (Fontana et al. 2004, 2008). Dietary analysis indicated an energy intake ~50% less than age-matched controls. In terms of body composition, the mean BMI of the males was 19.6 ± 1.9 kg/m^2 with an extremely low percent body fat of ~7%. Atherosclerosis risk factors including total cholesterol, LDL-c, HDL-c and triglycerides fell within the 50th percentile of values for people in their age-group. This report provides further evidence that longer term CR is highly effective in lowering the risk of developing coronary heart disease and other age-related co-morbidities. It remains to be seen if the CRONIES live longer than their age and sex matched counterparts.

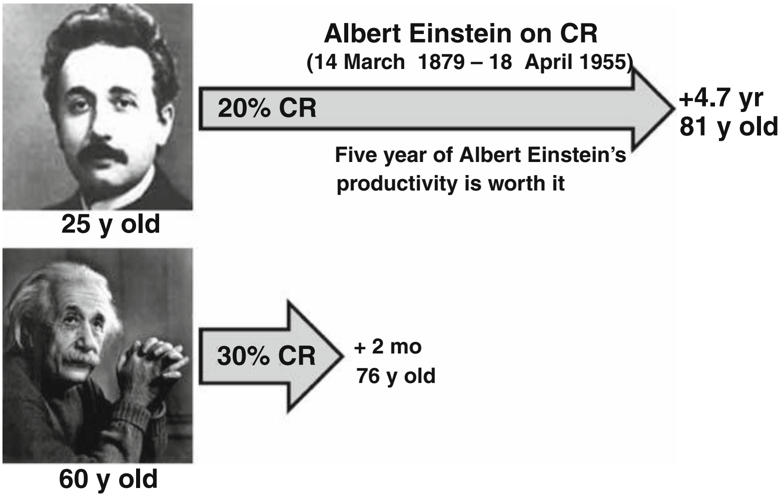
Albert Einstein on CR
(14 March 1879 – 18 April 1955)
20% CR
+4.7 yr
81 y old
Five year of Albert Einstein's productivity is worth it
25 y old
30% CR
+ 2 mo
76 y old
60 y old

Fig. 16.5 How can caloric restriction impact lifespan in humans? By extrapolating the data from rodents to humans (Merry 2000), one can predict the potential effect of caloric restriction in humans (Redman and Ravussin 2007). As an example, if Albert Einstein started a 20% CR diet at 25 years of age, he could have increased his life by approximately 5 years. On the other hand, undertaking a 30% CR diet 45 years later (age 60) would have extended his life by only 2 months. Therefore, CR needs to be initiated early in adult life to significantly increase life expectancy

16.8 Conclusions

While the rodent and primate data indicate that lifespan extension is possible with CR, collective analysis of the rodent data suggest that the intensity and onset of CR required to induce these effects is probably not suitable for many individuals (Redman and Ravussin 2007). Epidemiological studies certainly support the notion that a reduced energy intake that is nutritionally sound improves age-associated health. While results of the first randomized trials of CR, albeit short in duration suggest a reduction in risk of age-related disease and improvements in some biomarkers of longevity, the ultimate effect of this intervention on lifespan in humans will probably never be determined in the scientific setting. In our short-term study, calorie restriction was not associated with the development of eating disorder symptoms, decreased quality of life, depressed mood, or cognitive impairment all probably indicating the feasibility and safety of CR in humans. However, it is a challenge for most individuals to practice caloric restriction in an "obesogenic" environment that is conducive to overfeeding. Only very few individuals will be able to practice a lifestyle of caloric restriction and probably benefit from it. There is therefore a need for the search of organic or inorganic compounds that mimic the biological effects of CR. If such compounds often called "CR mimetics" such as resveratrol (Baur et al. 2006; Pearson et al. 2008) prove viable in humans, individuals for the most part will opt to enjoy the effects of anti-aging via a "pill" rather than CR.

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Chapter 17
Calorie Restriction and Cancer: An Update

Robin K. Minor, R. Michael Anson, and Rafael de Cabo

17.1 Introduction

Thanks to advances in medical care the average human life expectancy at birth is approaching 80 years, a gain of about 30 years compared with life expectancy at the turn of the twentieth century (Oeppen and Vaupel 2009). The same time span has also seen a shift in the causes of mortality such that the likelihood of dying prematurely from an infectious disease in a developed country has become quite low, while in contrast the likelihood of dying of a chronic disease has become relatively high. This has occurred despite major advances in the diagnosis and treatment of chronic diseases and currently heart disease, cancer, stroke, and chronic lower respiratory diseases are the leading causes of death in industrialized countries as well as many developing countries (Jemal et al. 2009; Eyre et al. 2004).

In the United States, cancer is the leading cause of death among women aged 40–79 and among men aged 60–79 (Jemal et al. 2009). The current lifetime probability of cancer diagnosis is about 44% for men and 37% for women. Among men, cancers of the lung and bronchus, prostate, and colorectum are the most common fatal cancers; among women cancers of the lung and bronchus, breast, and colorectum are the most deadly. In total, about 562,340 Americans are projected to succumb to cancer in 2009, corresponding to more than 1,500 deaths per day (Jemal et al. 2009).

Calorie restriction (CR), reduced calorie intake with sufficient micronutrient intake, is the only known non-genetic intervention which consistently increases both the mean and maximum lifespan across a broad spectrum of experimental models (Weindruch and Walford 1988). Implementation of CR in early to mid life maintains an organism's youthful physiology relative to age-matched ad libitum (AL) fed controls such that CR animals exhibit less age-associated loss of function and live significantly longer (Weindruch and Walford 1988).

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In this respect cancer and aging share an important commonality – both can be delayed by CR. In fact, while the lifespan-extending properties of CR were not well appreciated until the 1930s (McCay et al. 1935), observations of CR's protective effects against transplanted and induced tumors had been made nearly two decades earlier (Moreschi 1909; Rous 1914). Initially there was some doubt as to whether calories were the key variable or whether some other component of the diet might actually control the effect. Studies comparing the restriction of specific nutrient classes including carbohydrates, lipids, vitamins, etc. confirmed that reducing calories is indeed the key to extending lifespan (reviewed in Masoro 2006). Regarding CR's effects on cancer, a meta-analysis evaluating protection from mammary tumors in mice by individual dietary components concluded that calorie intake was the critical variable accounting for the antitumor effects of the different regimens (Dirx et al. 2003).

In the century or so since the effect of calorie intake on cancer was first noticed (Moreschi 1909; Rous 1914), progress has been sporadic. Each new technological advance has allowed incremental progress to be made, but a full understanding of the effect remains to be determined. Most recently, advances in molecular genetics have allowed new inroads to be made into the mechanisms by which calorie intake influences cancer, an aspect of the field on which this chapter will focus heavily. In addition, only recently have long-term, controlled experiments in primates – including humans – been possible, and the review will conclude with a consideration of these studies.

17.2 Molecular Mechanisms of Cancer Protection Through Calorie Restriction

Seeking "the" mechanism by which CR increases cancer resistance presupposes that only one mechanism is involved. While this is possible, it may well be that different mechanisms act during initiation, promotion, and progression. For example, various hormonal pathways have been shown to change their activity in response to CR, and these changes are relevant to cancer protection. However, the effect of each pathway may depend upon the type of cancer. Glucocorticoids, for example, appear to play a major role in protection by CR from skin carcinogenesis (Birt et al. 2004), but not in protection by CR from mammary carcinogenesis (Jiang et al. 2004). Insulin-like growth factor (IGF-1) may play a wider role (Moore et al. 2008), and yet CR protects against mammary carcinogenesis even when IGF-1 levels are increased by infusion (Zhu et al. 2005).

Maintenance of DNA stability is a fundamental and continuous challenge faced by every cell. Genomic instability is generally recognized as a nearly universal feature of both cancer and aging, fueling efforts to understand these enigmatic processes through their convergence (Finkel et al. 2007). Although CR has been shown to exert most of its effect during the promotion stage of tumorigenesis, there is evidence that it may be somewhat protective against DNA damage, and thus at least somewhat protective even against initiation (Azab et al. 2009; Garcia et al. 2008).

Furthermore, apoptosis is increased in many tissues, and so the opportunity for a damaged cell to survive long enough to reproduce is decreased.

CR is extremely protective even after initiation, however, and many studies over the years have used animals with implanted tumors as a model system. Normal cells possess mechanisms to prevent inappropriate growth. Cancer-inducing mutations must allow the cell to circumvent each of these control mechanisms. These mechanisms include cell-cycle checkpoints which ensure that division does not endanger the integrity of the genetic information, as it would (for example) if mitosis were to begin prior to completion of DNA synthesis. Other mechanisms for growth control include responses to cell-cell contact and to hormonal signals. The list of such mechanisms is fairly long, but the controls which seem most likely to be directly influenced by caloric intake are those which allow the cell to determine, directly or by responding to hormonal signals, whether raw materials and energy are available for growth.

CR has been extensively studied in animal models and has consistently shown beneficial effects on longevity and carcinogenesis across a variety of species (Kritchevsky 2002; Hursting et al. 2010). As is the case for lifespan extension, the mechanisms behind the protective effects of CR against cancer remain unknown. Elucidation of the molecular pathways through which CR exerts its protective and antiproliferative effects would allow for targeted treatment of specific cancers and greater outcome of success. The following sections describe studies that have shed light on possible molecular links between CR and cancer.

17.2.1 Nrf2

Our laboratory has focused on the NF-E2-related factor 2 (Nrf2) pathway, one of the mechanisms through which CR acts to upregulate the expression antioxidant enzymes (Hyun et al. 2006). This pathway has been studied for years, and particularly its induction by sulforaphane (a phytochemical abundant in cruciferous vegetables), for its inhibition of tumorigenesis (Juge et al. 2007). We demonstrated that the Nrf2 pathway is critically involved in CR's anticancer effects by showing CR is not protective against chemically-induced tumorigenesis in mice lacking Nrf2 (Pearson et al. 2008b). In fact, the Nrf2-knockout mice developed tumors more readily in response to carcinogen exposure than did control mice. A reduction in antioxidant expression downstream of Nrf2 could markedly increase DNA damage and spontaneous cancer incidence, and indeed NQO1 (a phase II detoxification enzyme) expression and activity was markedly reduced in the Nrf2 knockout mice on CR compared with control CR animals (Pearson et al. 2008b).

17.2.2 PI3K

Phosphoinositide 3-kinase (PI3K), a known regulator of cell growth, has recently been identified as another potential molecular mechanism for CR's antitumor effects (Kalaany and Sabatini 2009). The researchers found that cells with activated PI3K signaling were resistant to CR's antiproliferative effects, and they hypothesized that activated PI3K may be activating AKT, another kinase, which in turn may act on targets that lead to enhanced cell growth and inhibition of apoptosis. Interestingly, one of AKT's downstream targets is Nrf2 (Keum et al. 2004; Sakamoto et al. 2009) and thus the indirect action of PI3K on Nrf2 may mediate at least part of its effect on tumorigenesis.

17.2.3 SIRT1

In lower eukaryotes, the silent mating type information regulation 2 (Sir2) deacetylase is proposed to mediate the health benefits of CR (Sinclair 2005). SIRT1, the mammalian ortholog of Sir2, is induced by CR in multiple tissues and has been shown to forestall chronic diseases associated with aging such as cardiovascular dysfunction (Pearson et al. 2008a). The role of SIRT1 in CR's anticancer effects was recently investigated by us and others in a beta-catenin-driven mouse model of colon cancer (Firestein et al. 2008). Specifically we found an induction of SIRT1 expression in the intestine under CR conditions and that ectopic induction of SIRT1 significantly reduced tumor formation, cell proliferation, and animal morbidity even without CR (Firestein et al. 2008). We also showed that SIRT1 can deacetylate beta-catenin and thereby suppress its ability to activate transcription and drive cell proliferation.

17.3 Human Relevance

As the incidence of obesity continues to increase in much of the developed world (Ogden et al. 2006), so too accumulates evidence that obesity is an important risk factor for several cancers (Pan and DesMeules 2009). Thus, CR may have direct applications to cancer prevention strategies in human populations and the rest of this review is dedicated to exploring the available literature on how CR could influence carcinogenesis in human beings.

Data from nonhuman primate models are inconclusive at this time but should provide valuable information in the near future. Thus far CR instituted in adult rhesus monkeys has not shown significant benefits to chromosomal stability (Moore et al. 2007). However, final results from several nonhuman primate studies that are currently under way will give researchers more clues about the effects of caloric restriction on cancer in humans within the next few decades (Ramsay et al. 2000; Mattison et al. 2007).

Regarding humans it has been suggested that caloric intake may be protective against cancer development because CR is known to have such potent antitumor effects in experimentally-induced animal models (Hursting et al. 2003). Indeed, some epidemiological evidence from populations surviving under low-calorie conditions have shown reduced cancer rates. For example, inhabitants of Okinawa, an island off mainland Japan, consumed fewer calories through the mid twentieth-century than their mainland neighbors and displayed lower death rates from cancer and vascular diseases (Willcox et al. 2007). Interestingly, modernization of Okinawa has resulted in normalization of the Okinawan diet with that of the rest of Japan, particularly among the young, and subsequent disease rates have risen in step (Miyagi et al. 2003). Another example comes from a retrospective study examining breast cancer incidence in women hospitalized for anorexia nervosa (Michels and Ekbom 2004). The anorexic women (i.e. CR with micronutrient insufficiency) were found to have lower incidences of breast cancer compared with the general population. Furthermore, if the anorexia occurred prior to a pregnancy, the protective effect was even more evident (Michels and Ekbom 2004).

17.4 Making CR More Attainable to Humans: Short-term Fasting and Every-Other-Day Feeding

In animal studies, CR usually entails food intake restriction ranging from 20 to 40% on a daily basis. Because such extreme dieting over the long term is likely to be unrealistic in humans, researchers are now examining the biological pathways affected by caloric restriction with the aim of developing preventive strategies to target cancer in a more palatable way suitable to practical application.

One such adaptation of CR to humans may be short-term CR, or fasting. In mice, short-term CR (30–50% CR over periods of 2–3 weeks followed by ad libitum feeding for an equal amount of time) has been shown to be protective against tumor formation in models for both prostate cancer and breast cancer (Cleary et al. 2007; Bonorden et al. 2009). Another mouse study has suggested that an even shorter bout of food restriction (in this case the mice were completely fasted with access only to drinking water) of just 2 days was able to block the toxicity of the drug etoposide to the mice such that they displayed normal behavior and regained weight more rapidly than fed controls (Raffaghello et al. 2008). Importantly, fasting was observed to block the negative effects of chemotherapy but not the antitumor activity of the drug.

Another adaptation of CR to humans may involve periodic fasting. Indeed, reducing overall caloric intake through intermittent periods of food restriction has been shown to be both attainable and beneficial to human health (Johnson et al. 2007; Williams et al. 1998). For example, overweight subjects participating in an 8-week study using alternate day caloric restriction (alternating daily between ad libitum intake and ~80% CR) lost weight and increased in measures of perceived energy and mood (Johnson et al. 2007). Also, several recent short-term interventions have examined every-other-day feeding in nonobese humans (Halberg et al. 2005; Heilbronn et al. 2005a, b) and found significant decreases in fat mass (Heilbronn et al. 2005b) and an increases in plasma adiponectin (Halberg et al. 2005).

Before extrapolating protocols which involve severe CR or fasting to humans, a caveat is in order. Thriving during a food shortage – even a short-term one – requires good glycemic control and the ability to adjust metabolism appropriately. Animals (or humans) with defects in these pathways may be at a disadvantage. This is evidenced by observations in mammals that defects in corticosteroid pathways or insulin/glucagon signaling preclude full adaptation to a CR diet (Pashko and Schwarz 1996; Garcia et al. 2008). In such cases death by cancer may be prevented, but complications resulting from poor glycemic control, or inadequate adjustments in the metabolism of protein, lipid, or other nutrients may nonetheless lead to the subject's premature demise.

17.5 Conclusion

To date, CR has been the most widely studied and most effective experimental strategy for increasing the survival of mammals (Weindruch and Walford 1988), and it is also the most potent, broadly acting dietary intervention for preventing carcinogenesis in experimental models (Hursting et al. 2003).

As researchers learn more about the mechanisms through which CR exerts its antiproliferative effects it may become possible to envision the discovery or development of cancer-prevention agents based on multiple CR pathways for enhancing the efficacy of cancer treatment. Research in animal models and nonhuman primates in particular will continue to shed light on the pathways through which CR can mitigate neoplasia in humans.

By virtue of the severity of traditional CR implemented in laboratory situations, further research into short-term and every-other-day feeding adaptations of the CR regimen may yield an alternative that may not only be more realistic in the human case but may even be more desirable than long-term food restriction.

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Chapter 18
Conclusion: Human Calorie Restriction and Anti-aging Therapy

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18.1 Food Intake Effect on Aging and Longevity

During the twentieth century improvements in public health, nutrition and medicine have increased human life expectancy at birth in 18 developed countries from 50.3 years in 1900 to 78.3 years in 2000 (Tiffen and Gittins 2004). In the early twenty-first century life expectancy in developed countries is continuing to increase by about 2 months in every year, due largely to medical interventions that are able to reduce deaths from cardiovascular disease and cancer. Also the number of centenarians is doubling every 10 years (Vaupel 1997). Thus life extension is occurring despite a growing epidemic of obesity due to overeating, which accelerates the onset of life-shortening diseases (Bessesen 2008). It has been known since the classical study by Clive McCay in 1935 that life-long reduction in food intake prolongs the life-span of the laboratory rat. This result has been replicated many times in different laboratories in short-lived animal models. Thus calorie restriction (CR) or reduced food intake in short-lived animals clearly inhibits the development of risk factors for age-related diseases, and thus delays their onset and prolongs life-span by up to 50%. Recently a 20-year longitudinal adult-onset calorie-restriction study in rhesus monkeys was reported to delay the onset of age-associated pathologies, reduce mortality and prolong life (Colman et al. 2009). Human studies of 20% CR over 2 years (Walford et al. 2002) to 6 years (Fontana et al. 2004) also show that food restriction, without malnutrition, reduces aging risk factors. In these studies CR had beneficial effects on many physiological and biochemical parameters linked to cardiovascular disease and diabetes such as body weight, blood pressure, blood cholesterol and blood glucose. Another study found that Okinawans, the longest-lived people on Earth, have been consuming 20–40% fewer calories than Americans over the last 40 years and have lived 4 years longer (Willcox et al. 2006). Similarly, women in the United States consume 25% fewer calories than men and live 5 years longer

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(Tiffen and Gittins 2004). Thus, long-term CR or lower food intake extends human life-span by only about 4–5 years. In addition to calorie intake, lifestyle factors also influence longevity. The major life-shortening factors in human lifestyle studies are smoking and obesity. A lifestyle study of 77,782 middle-aged women followed over 24 years showed that eating a healthy diet, high in fruit and vegetables, maintaining normal body weight and moderate exercise, plus not smoking added 10 years to life expectancy (van Dam et al. 2008). In recent years the US population has developed a healthier lifestyle of less smoking, lower serum cholesterol, and fewer drunk drivers. Most age-related illnesses in humans can be delayed by preventative health strategies. Survival to age 90 years is increased by the absence of smoking, diabetes, obesity, high blood pressure and by exercise (Yates et al. 2008). A low calorie intake in food diminishes the risk of diabetes, obesity and high blood pressure, but there are other factors in life style such as exercise and smoking that affect life expectancy.

18.2 Mechanism of the Anti-aging Action of Calorie Restriction

Calories are obviously important determinants of aging, disease development and longevity (Masoro 2005). There is, however, no general agreement on the mechanism of the anti-aging effect of food restriction. The actions of CR on physiological aging and disease onset may be different. An early theory emphasised the reduction in growth. Later theories stressed other factors such as the decrease in body fat, reduced metabolic rate, decreased calorie intake, reduced oxidative damage to DNA in mitochondria, reduced inflammation, increased mild stress (the hormesis theory), amongst others. There are studies suggesting that the protein content of the diet may affect aging processes (Ayala et al. 2007). CR also exerts anti-inflammatory and anti-oxidative actions which affect the body's ability to respond to bacterial challenge (Chung et al. 2001).

The oxidative stress theory of aging is a well-studied theory of aging (Sohal et al. 2002; Hunt et al. 2006). In normal living tissues, oxygen reacts with energy in various nutrients, such as carbohydrates, proteins, fats and vitamins. A small percentage of the oxygen molecules are highly reactive and cause damage to macromolecules, that increase aging in tissues. The role of reactive oxygen species (ROS) in determining the rate of aging remains contentious. Energy and oxygen in the tissues interact with numerous endogenous and exogenous biochemicals, including metabolites, polyphenols, resveratrol, sirtuins, other enzymes, hormones, genes, to name just a few. Thus the anti-aging actions of CR by reducing oxidative damage may involve many different systems and tissues in the body.

18.3 Physiological and Biochemical Action of Calorie Restriction

A 20% reduction in food intake (that is 20% CR) in human subjects acting over 2–6 years has beneficial effects on many physiological and biochemical parameters linked to cardiovascular disease and diabetes such as reductions in body weight, blood pressure, blood cholesterol and blood glucose (Walford et al. 2002; Fontana et al. 2004).

In the physiological and biochemical control of body functions the genes, brain, nerves, hormones and liver are important regulators, and appear to play significant roles in the anti-aging actions of reduced calorie intakes. The liver has a central role in metabolism and detoxification, and by this means may affect the anti-aging actions of CR (Le Couteur et al. 2009). There are significant age-related changes in liver mass, blood flow and hepatocyte and sinusoidal cell morphology. The effects of caloric restriction in the liver are beneficial but caloric restriction does not just simply reverse or delay age-related cellular changes. Instead caloric restriction appears to act via discrete cellular mechanisms such as sirtuin pathways that impact cellular bioenergetics, apoptosis and other cellular functions. The age-related impairment of the hepatic metabolism of lipoproteins predisposes to cardiovascular disease. The liver has a pivotal coordinating role when dietary intake is reduced, and many of the effects of caloric restriction are mediated by the liver. CR reduces the secretion of many hormones, including insulin like growth factor 1 (IGF1) in animals but not humans (Fontana et al 2008). IGF1 secreted by the liver increases longevity in many different strains of mice (Yuan et al. 2009).

Studies in dwarf mice show that a lack of certain pituitary hormones, via actions on specific genes, inhibits growth and prolongs life-span (Bartke and Brown-Borg 2004). If these mice are then placed on CR a further life extension occurs. When hypophysectomized rats receive 1 mg cortisone per week and eat the same amount of food as CR rats, they age more slowly and live longer than CR rats (Everitt 1971). This suggests that adrenocortical hormones have anti-aging effects. CR rats have raised levels of corticosterone. This lends support to the hormesis theory of calorie restriction, namely that low level stress, which releases adrenocortical hormones may be the basis of the anti-aging effects of CR (Masoro 1998). Raised levels of cortisol and ACTH are found in the blood of centenarians (Genedani et al. 2008). Similarly, some long-living mammals such as dwarf mice, also have elevated plasma corticosterone concentrations.

18.4 Genes and Aging

A number of genes have been identified that contribute to aging and longevity in various species. In humans, there is a growing literature showing that genes play significant roles in aging and longevity (Barzilai and Shuldiner 2001). The hereditary influence is most apparent in the oldest-old. Siblings of centenarians are 3–4 times more likely to survive into their nineties. The majority of longevity genes in humans are involved in the insulin/insulin-like growth factor (IGF) pathway. A polymorphism in the FOXO3A gene, for example, has shown an association with longevity in a study of centenarians (Flachsbart et al. 2009).

18.5 Resveratrol

There is evidence in mice and lower organisms that polyphenols, such as resveratrol, have similar anti-aging actions as CR, by activating sirtuin enzymes and increasing resistance to oxidative stress. Polyphenols act like CR in helping cells to resist adverse or stressful conditions.

When mice were fed a high calorie diet they became fat and ill, but when another group fed the same diet were concurrently given a daily dose of resveratrol they remained healthy and survived longer (Baur et al. 2006). Resveratrol is found in berry fruits such as grapes, blueberries and in red wine. Anti-aging medicine in the future should be more effective, considering not only novel pharmaceuticals, but also improved nutrition, physical exercise and beneficial lifestyle factors.

18.6 Obesity

Food intake has been rising since the 1960s, with the greatest increase in United States (Tiffen and Gittins 2004). High food intake leads to overweight (body mass index, BMI of 25 or more) and obesity (BMI 30 or more). Obesity, has increased markedly since the 1980s. and is now in epidemic proportions globally. In Australia 20% of adults were obese in 2001. In USA 11% of children are obese (Dehghan et al. 2005). Overweight and obesity increase the risk for type 2 diabetes, heart disease, high blood pressure, high blood cholesterol and cancer. Obesity is second to tobacco smoking as the preventable cause of death in United States (Brawer et al. 2009). As a result of the obesity epidemic increasing the incidence of diseases which shorten life the rise in life expectancy that occurred during the twentieth century may come to a halt in mid twenty-first century (Olshansky et al. 2005).

Prevention of obesity is urgently required starting with a focus on young people (Catford and Caterson 2003; Dehghan et al. 2005). Both overconsumption of calories in food and reduced physical activity are causes of childhood obesity. Some other factors in children are increased television watching, plus TV advertising promoting high energy foods and soft drinks. Fruit and vegetable consumption by children has decreased over the last 20 years (Waters and Baur 2003). There has been little or no progress so far in obesity prevention in childhood by changes in lifestyle. However, pediatric obesity can be successfully treated by bariatric surgery (Treadwell et al. 2008). In a well-controlled 5 year- survival study, bariatric surgery significantly reduced mortality by 89% (Christou et al. 2004). Bariatric surgery has successfully treated severe obesity over a 10 year period, resulting in long-term weight loss, and restoration of body functions to normal apart from hypercholesterolemia (Sjostrom et al. 2004).

18.7 Cardiovascular Disease

Cardiovascular disease (CVD) is a major cause of morbidity and death in humans. Therefore life extension should occur if calorie restriction were to slow the progression of atherosclerosis. A 20% reduction in food intake over 6 years in 18 middle-aged subjects compared with 18 controls reduced certain risk factors for atherosclerosis such as body weight, blood pressure, serum cholesterol and triglyceride (Fontana et al. 2004). CR also exerts anti-inflammatory and anti-oxidative effects that preserve the health of the cardiovascular system (Holloszy and Fontana 2007). A high intake of fruit and vegetables rich in antioxidants is believed to reduce the risk of CVD and probably extend life (Mirmiran et al. 2009).

18.8 Diabetes

Type 2 diabetes is the most common metabolic disease in the world and is becoming a global epidemic, predicted to afflict 5% of the population by year 2025, secondary to population aging and obesity (Alberti et al. 2004). In middle aged, overweight subjects with impaired glucose tolerance, it is possible to prevent type 2 diabetes by changes in lifestyle over a 3-year period, such as reductions in body weight, total fat intake and saturated fat intake, with increases in fiber intake and physical activity (Tuomilehto et al. 2001). Such a study cannot determine whether weight loss, energy restriction, or physical activity are more important. Type 2 diabetes in childhood can be successfully treated with a very low calorie diet instead of pharmacologic agents such as insulin or metformin (Willi et al. 2004). In a 6-year 20% calorie restriction study on middle-aged healthy subjects blood sugar levels were reduced by 15% (Fontana et al. 2004). The consumption of green leafy vegetables and fruit was associated with a lower risk of diabetes in an 18 year study of 71,346 female nurses in middle age originally free of cardiovascular disease, cancer and diabetes (Bazzano et al. 2008).

18.9 Cancer

Cancer incidence rises steeply with age, with more than half of all cancers diagnosed in the over- 65 year group (Goodwin and Brodwick 1995). Diet is second to tobacco smoking as a preventable cause of cancer. Long-term calorie restriction is the most potent intervention for preventing cancer onset in experimental animals (Hursting et al. 2003). In humans overeating leading to overweight and obesity results in a higher incidence of common and less common malignancies (Renehan et al. 2008). There is evidence that fruit and vegetable diets may reduce the risk of cancer (Riboli and Norat 2003).

18.10 Dementia

Increasing age is the strongest epidemiological risk factor for dementia. There is evidence that specific nutrients and certain dietary patterns may affect the risk of developing dementia (Whalley et al. 2004). High caloric and fat intakes are associated with increased risk of dementia, while fish and seafood may reduce the risk.

18.11 Longevity

Studies conducted on lower animals such as rats or mice have repeatedly shown that a nutritious diet with a low calorie content will prolong life by up to 50% (Weindruch and Walford 1988).

Recently it was reported in the rhesus monkey, a primate close to humans, that 20 years of 30% CR significantly prolonged life (Colman et al. 2009). An equivalent study in humans would have to run for about 100 years for the longevity effect of CR to be assessed. On indirect evidence it is estimated that CR would increase human life by only about 5 years (Willcox et al. 2006). In the early twenty-first century successful medical research into the cause and treatment of diabetes, obesity, cardiovascular disease, respiratory disease and cancer should continue to extend human life at the rate of about 2 months in every year.

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